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Review

Methods for the genetic manipulation of marine bacteria



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ABSTRACT

Genetic manipulation of bacteria is a procedure necessary to obtain new strains that express peculiar and defined genetic determinants or to introduce genetic variants responsible for phenotypic modifications. This procedure can be applied to explore the biotechnological potential associated with environmental bacteria and to utilize the functional properties of specific genes when inserted into an appropriate host. In the past years, marine bacteria have received increasing attention because they represent a fascinating reservoir of genetic and functional diversity that can be utilized to fuel the bioeconomy sector. However, there is an urgent need for an in-depth investigation and improvement of the genetic manipulation tools applicable to marine strains because of the paucity of knowledge regarding this. This review aims to describe the genetic manipulation methods hitherto used in marine bacteria, thus highlighting the limiting factors of the different techniques available today to increase manipulation efficiency. In particular, we focus on methods of natural and artificial transformations (especially electroporation) and conjugation because they have been successfully applied to several marine strains. Finally, we emphasize that, to avoid failure, future work should be carried out to establish tailored methodologies for marine bacteria.

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1. Introduction

Microbial diversity present on the Earth is endless. Marine microorganisms are a fascinating reservoir of genetic and functional diversity, and their products could be utilized in many different biotechnological sectors. For instance, they can produce bioactive

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natural compounds, enzymes useful for industrial applications, or pharmaceutical agents with anticancer, antimicrobial, or anti-inflammatory properties [1,2,3,4,5,6,7]. However, many marine microorganisms remain unutilized because of the lack of efficient isolation and/or cultivation methods and the consequence of the inadequate genetic manipulation procedures available compared to the those available for the culturable microorganisms [8,9]. The importance of innovative and efficient methods for cultivating marine bacteria for the biodiversity pipeline has been thoroughly examined [8]. Therefore, in this review, we consider the procedures involved in genetically manipulating marine strains. Genetic manipulation (or engineering) of microorganisms is generally applied to obtain new strains that express additional genetic properties or to introduce genetic variants with phenotypic alterations [10]. Particularly, one of the most interesting applications in the field of marine microbiology is the utilization of genetic manipulation methods to (i) explore the enormous undiscovered source of genetic information associated with the environmental samples through the screening of metagenomic libraries expressed in suitable culturable marine hosts [11] and to (ii) study the biotechnological potential of culturable marine strains by investigating the expression and function of genes of interest [12]. Heterologous expression of genes directly from environmental metagenomes can be seen as an approach in principle that can overcome the limitations because of the poor culturability of marine strains. However, this technique has several disadvantages, as reviewed by Lam et al. [13], and cannot entirely substitute the biotechnological application of novel and manipulated marine microbial isolates, especially regarding the poor efficiency of conventional hosts in expressing genes derived from distantly related microorganisms. Heterologous expression of metagenome-captured genes in novel, nonconventional marine hosts could nevertheless improve the success of the approach. Efficient gene transfer systems are therefore required to improve the utilization of the biotechnological potential associated with marine microbial strains, also considering advantages from the recent technological improvements such as the next-generation DNA sequencing techniques and single-cell genome analysis.

Prokaryotic cells naturally acquire exogenous DNA through different pathways including transduction through bacteriophage infection [14, 15], conjugation mediated by cell–cell contact [16], or directly from the environment through a natural physiological state of competence developed by the bacterium [17]. Researchers utilized these natural mechanisms of horizontal gene transfer (HGT) for cell manipulation. In general, many studies investigated HGT through transformation and conjugation rather than transduction. With regard to transduction and considering marine environments, recent interest has been directed toward a particular genetic exchange mechanism that is active in natural environments and is mediated by virus-like elements called as gene transfer agents (GTAs) and toward the evaluation of the role of phage in spreading antibiotic resistance genes or in shaping the microbial evolution and ecology [18,19,20,21].

Over the past decades, special attention has been focused on the gene transfer by bacteria in a natural competent state [22,23], a time-limited physiological state specifically developed by some species in response to different external factors. Heretofore, more than 87 bacterial species have been reported as being naturally competent to take up extracellular genetic material [17,24]. The environment in which bacteria thrive plays an important role in their adaptation and evolution, which also influences their genetic transformation efficiency. For instance, natural transformation in the marine environment was demonstrated to occur more frequently in sediments than in the water column owing to the capability of sediment particulates to protect DNA from enzymatic degradation [23,25,26]. Meibom et al. [23] reported that the presence of chitin, which is one of the most abundant biopolymers in aquatic environments, promotes the development of natural competence in *Vibrio* strains including the two pathogens *Vibrio cholerae* and *Vibrio vulnificus*. Even under extreme marine

environments such as the brines of the deep hypersaline anoxic basins (DHABs) of the Mediterranean Sea [27], naked DNA was demonstrated to be preserved and to maintain the transforming potential of competent cells [28]. The brines of DHABs constitute an extremely aggressive environment for cells and macromolecules by providing hypersaline, anoxic, and strong reducing conditions. Nevertheless, plasmid DNA incubated in the brines of different DHABs up to 32 d retained the capacity to transform the naturally competent *Acinetobacter baylyi* BD413 cells [28].

To insert DNA into bacteria, different artificial methods have been developed, including chemical and physical techniques. Because homologous recombination of exogenous DNA in a host cell is limited to sequences with high DNA similarity, shuttle vectors are preferred to compel DNA entry into bacterial cells [29]. Plasmids are extrachromosomal DNA elements that are usually transferred in nature to both closely and distantly related bacterial species according to their replication origin [30,31]. They are generally used as shuttle vectors in electroporation and conjugation-based procedures owing to their relative ease of manipulation. Certain bacteria remain nevertheless recalcitrant to internalize and express exogenous plasmid DNA [32]. Electroporation is a known, easy, and rapid tool to successfully transform a large range of bacteria, but it is still unsuitable for several marine species [33]. In the following sections, we aim to summarize the currently used methods for the genetic manipulation of marine bacteria and to emphasize the limitations that could prevent the success of the methods. Specifically, we consider natural and artificial transformations and conjugation-based procedures because these methods have been adopted in the majority of the studies on manipulating marine bacteria (Fig. 1, Table 1).

2. Natural competence of marine bacteria

Mechanisms underpinning natural genetic transformation have been described in several bacterial strains, i.e., the human pathogenic strains *Haemophilus influenzae* and *Streptococcus pneumoniae* and the model organism of gram-positive bacteria *Bacillus subtilis* [34]. Moreover, natural transformation has been studied in relation to the genome evolution in the environmental natural-competent species *A. baylyi* [35]. In aquatic environments, natural transformation occurs favorably in the presence of free DNA [36] and naturally competent cells [37]. Nevertheless, only a limited number of studies have investigated this process in marine isolates, considering only few species [23,38]. In 1989, Stewart and Sinigalliano [38] showed for the first time that natural transformation could occur in a marine bacterium and regarded the strain *Pseudomonas stutzeri* ZoBell as the first naturally transformable marine model. Frischer et al. [39] focused on the chemical and physical factors that affect competence in the marine strain *Vibrio* WJT-1C (later identified as *Pseudomonas* sp. [40]). In this strain, natural competence arises at the early exponential phase and lasts almost 10 d. The authors verified that its transformation efficiency was not susceptible to the environmental variations typical of the estuarine environment, such as temperature, nutrient concentration, and salinity shifts, thus suggesting that this environment represented, for the considered strain, a suitable niche of natural transformation [39].

Many studies on natural transformation focused on the genus *Vibrio* [23,41] (Table 1 and Table 2) and, particularly, on *V. cholerae*. In addition to being a human pathogen, *V. cholerae* is an inhabitant of the aquatic environment [42], where it has been described as a good colonizer of chitin-based surfaces [43]. Chitin is the major constituent of copepod exoskeletons, crab shells, and diatoms [44,45]. It represents an important nutrient source for chitinolytic bacteria including those belonging to the family *Vibrionaceae*. Indeed, they can break the chitin down into soluble subunits of N-acetylglucosamine (GlcNAc) and chitobiose (GlcNAc₂) and use it as a sole source of carbon [23,44,45, 46]. Meibom et al. [23] discovered that chitin plays another intriguing

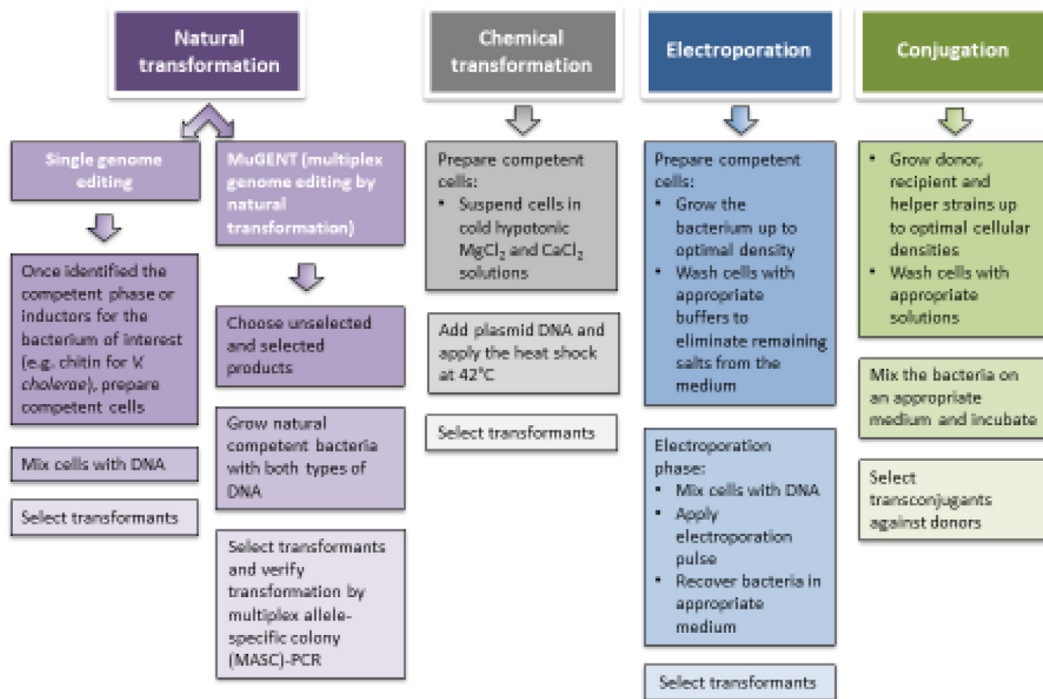


Fig. 1. Schematic representation of the workflow for the genetic manipulation of bacteria by natural transformation (including single and multiple genome editing), chemical transformation, electroporation, and conjugation.

role in *Vibrio* by making it competent to uptake exogenous DNA. After this discovery, the molecular mechanisms behind the competence state in *V. cholerae* were explored in detail [47,48,49,50,51]. In particular, it has been shown that the bacterial regulatory network harmonizes the response of cells to starvation, hence activating competence through three extracellular systems. The first system responds to chitin and activates the transcription of the regulatory gene *tfoX* [52], which in turn regulates the activation of enzymes involved in the degradation of chitin into soluble forms. Then, the TfoX-induced genes, as well as other components, regulate the production of the components of the DNA-uptake machinery including a central type IV pilus structure [23]. The second system responds to high cellular density. A quorum-sensing regulator

promotes the expression of the *hapR* gene [23,51,53,54], which represses the expression of the extracellular nuclease Dns and regulates the activation of genes necessary for natural transformation [55,56]. A third extracellular nucleoside system allows the expression of the protein CytR, which functions as an additional positive regulator for competence acquisition [57]. The natural competence state has also been identified among isolates belonging to other *Vibrio* species such as *Vibrio parahaemolyticus* [58], *V. vulnificus* [59], and *Vibrio fischeri* [60].

Although chitin is able to induce competence in different *Vibrio* species, the competence apparatus does not seem to be conserved. Comparison of genomic sequences showed that an additional TfoX-like protein, designated as TfoY, is present in

Table 1

Gene transfer systems used for marine bacteria. Advantages and criticisms of the procedures are listed, together with the names and references of selected bacterial species on which they have been successfully applied.

Gene transfer system	Advantages	Criticisms	Examples of bacterial species/groups on which successfully applied
Natural transformation	<ul style="list-style-type: none"> – Easy procedure; – Well-established method for <i>Vibrio</i> strains (chitin-based protocol) 	<ul style="list-style-type: none"> – Time-consuming procedure; – It requires natural competent cells; – It requires large amounts of DNA 	<ul style="list-style-type: none"> – <i>Vibrio cholerae</i>, <i>V. fischeri</i>, <i>V. parahaemolyticus</i>, <i>V. vulnificus</i> [23,58,59,61]. – <i>Pseudomonas stutzeri</i> ZoBell [38] – <i>V. natriegens</i> [73]
Chemical transformation	<ul style="list-style-type: none"> – Easy procedure 	<ul style="list-style-type: none"> – Dependent on the restriction endonucleases of the recipient bacterium; – It requires chemical competent cells 	
Electroporation	<ul style="list-style-type: none"> – Well-established and quick method; – Applied on a broad range of species including bacteria and archaea; – High efficient transformation procedure; – It requires small amount of DNA 	<ul style="list-style-type: none"> – Low success rate; – Dependent on the restriction endonucleases of the recipient bacterium; – Affected by the presence of salt and composition of media and buffers; – Affected by topology, size, and concentration of DNA – Affected by field strength; – It requires electrocompetent cells 	<ul style="list-style-type: none"> – <i>V. cholerae</i>, <i>V. parahaemolyticus</i>, <i>V. alginolyticus</i>, <i>V. natriegens</i>, <i>V. vulnificus</i>, <i>V. anguillarum</i> [70,71,72,73,85]. – <i>Pasteurella piscicida</i>, <i>Roseobacter</i> clade; <i>Pseudoalteromonas</i> PS1M3, <i>Caulobacter crescentus</i>, <i>Synechococcus</i> sp. CC9311, <i>Halomonas</i> sp. O-1 [72,73,77,81,82,83,84,96]
Conjugation	<ul style="list-style-type: none"> – Well-established method; – It bypasses restriction endonuclease barrier of the bacterial host 	<ul style="list-style-type: none"> – Laborious (time-consuming method); – Possible issues in the selection of transconjugants after mating 	<ul style="list-style-type: none"> – <i>V. cholerae</i>, <i>V. vulnificus</i>, <i>V. parahaemolyticus</i>, <i>V. alginolyticus</i> [127]. – <i>Pseudoalteromonas</i> sp. SM9913, <i>Roseobacter</i> clade, <i>Prochlorococcus</i> MIT9313, <i>Marinobacter adhaerens</i> HP15 [33,72,127,128,132,133].

Table 2
List of selected genetically manipulated marine bacteria mentioned in the review.

Bacterial species or strains	Vector ^a	Methods of manipulation	Characteristics of the introduced DNA	Expressed genes	Technological interest	Ref.
<i>Bacillus marinus</i> B-9987	– Unmethylated p3101GFP (derivative plasmid of pHT3101; <i>Emr</i> ^r)	Electroporation	Circular plasmid	Gfp	Developing an efficient electroporation method for marine-derived <i>Bacillus</i> strains	[77]
	– Unmethylated p3101SFP (derivative plasmid of pHT3101; <i>Emr</i> ^r)	Electroporation	Circular plasmid	Expression of the <i>sfp</i> gene, a gene essential for the biosynthesis of polyketides and nonribosomal peptide antibiotics	Developing an efficient electroporation method for marine-derived <i>Bacillus</i> strains	[77]
<i>Dinoroseobacter shibae</i> DFL12 ^T	pEX18Δdnr::Gmr (<i>Gm</i> ^r ; <i>Amp</i> ^r ; <i>sacB</i> ; <i>lacZ</i> ; suicide vector)	Conjugation with <i>E. coli</i> ST18	With homology to the chromosome	Replace <i>dnr</i> gene with the gentamicin resistance gene	Obtaining a gene knockout to study the influence of the regulator Dnr on the bacterial growth under denitrifying conditions	[72]
<i>Fischerella</i> and <i>Chlorogloeopsis</i> strains	pRL25C (and eventually helper strains pRL443, pRL623),	Electroporation, conjugation, and DNA bombardment	Circular plasmid	Antibiotic resistance genes	Developing a genetic manipulation system for the strains	[97]
<i>Halomonas</i> sp. O-1	– pBBR1MCS (Broad host range plasmid; MCS; <i>lacZα</i> ; <i>Cm</i> ^r) – pBBR1MCS-5 (Broad host range plasmid; MCS; <i>lacZα</i> ; <i>Gm</i> ^r)	Electroporation	Circular plasmid	Antibiotic resistance genes	Developing an electroporation method for <i>Halomonas</i> sp. transformation	[83]
<i>Marinobacter adhaerens</i> HP15	pBBR1MCS (Broad host-range; mob; <i>Cm</i> ^r); pSUP106 (IncQ; mob; <i>cos</i> ; <i>Cm</i> ^r ; <i>Tet</i> ^r); pBBR.EGFP (derivative of pBBR1MCS; <i>egfp</i>); pRL27 (IncX oriT, Tn5; <i>Km</i> ^r); pITM1 and pITM2 (derivatives of pBBR1MCS)	Electroporation	Circular plasmid	Antibiotic resistance genes, Gfp	Developing a genetic manipulation system for the strain	[132]
	pBBR1MCS; pSUP106; pAS7 (derivative of pGEM®-T Easy); pAS8 (derivative of pGEM®-T Easy);	Conjugation	Circular plasmid	Antibiotic resistance genes	Developing a genetic manipulation system for the strain	[132]
<i>Prochlorococcus</i> sp. MIT9313	pRL153 (RSF1010 derivative, <i>Km</i> ^r)	Conjugation with <i>E. coli</i> 1100-2	Circular plasmid	Gfp	Studying conjugation-based methods in <i>Prochlorococcus</i>	[133]
	pRL27 (<i>Km</i> ^r ; mini-Tn5; Ori R6K)	Conjugation with <i>E. coli</i> BW19851	Circular plasmid	Insertional mutagenesis through Tn5	Studying conjugation-based methods in <i>Prochlorococcus</i>	[133]
<i>Pseudoalteromonas</i> sp. SM9913	pMT suicide vector (constructed from pOriT-4Em; <i>sacB</i> ; <i>Amp</i> ^r ; <i>Emr</i> ^r ; flanking regions of <i>epsT</i>)	Conjugation with <i>E. coli</i> ET12567	With homology to the chromosome	Knockout the <i>epsT</i> gene	Developing a genetic manipulation system for <i>Pseudoalteromonas</i> sp. SM9913	[128]
<i>Pseudoalteromonas</i> sp. BSI20429	pWD (derived from the ligation of pSM429 and pUC19; Ori pBR322, <i>Cm</i> ^r)	Electroporation	Circular plasmid	Expression of a cold-adapted cellulase	Developing a heterologous expression system for <i>Pseudoalteromonas</i>	[116]
<i>Roseobacter</i> clade sp.	pBBR1MCS	Electroporation	Circular plasmid	Antibiotic resistance genes	Developing a genetic manipulation system for the strains in this clade	[72]
	IncQ-plasmids pRSF1010, pMMB67EH, and pBBR1MCS derivatives	Conjugation with <i>E. coli</i> ST18 (a hemA mutant of <i>E. coli</i> S17 λ-pir)	Circular plasmid	Antibiotic resistance genes	Developing a genetic manipulation system for the strains in this clade	[72]
<i>Synechococcus</i> sp. CC9311	Integrative plasmid pMD18TmpeV	Electroporation	Circular	Kanamycin resistance and disruption of the <i>mpeV</i> gene	Developing a genetic manipulation system for the strain	[84]
<i>Vibrio cholerae</i>	–	Natural competence	With homology to the chromosome	Antibiotic resistance genes	Studying natural competence in <i>V. cholerae</i> strains	[23]
<i>Vibrio fischeri</i>	–	Natural competence	Chromosomal, plasmid or linear forms of DNA	Antibiotic resistance genes	Studying natural competence in <i>V. fischeri</i> and developing a genetic manipulation system for this species	[60]
<i>Vibrio harveyi</i> ORM4	pVSV102 (GFP; <i>Km</i> ^r)	Conjugation with <i>E. coli</i> CC118 λpir and <i>E. coli</i> strain DH5α	Circular plasmid	Gfp	Studying the disease progression phases of <i>V. harveyi</i> ORM4 in abalones	[115]
<i>Vibrio natriegens</i>	–	Natural competence (MuGENT)	Linear DNA	Antibiotic resistance genes and other targets (e.g., genes in the poly-β-hydroxybutyrate biosynthesis operon)	Developing a genetic manipulation system for the strain	[140]
<i>V. natriegens</i>	pACYC184; pBR325, and pET vectors; pUC19; plasmids with the RK2	Electroporation; chemical transformation; conjugation	Circular plasmid	Antibiotic resistance genes, Gfp	Developing a genetic manipulation system for the strain (also utilizing the Cre-loxP system)	[73]
<i>V. natriegens</i>	pDM4 (carrying the flanking regions of target genes; <i>sacB</i> , ori R6K; <i>Cm</i> ^r)	Conjugation with <i>E. coli</i> S17-1 λpir	With homology to the chromosome	Chromosomal deletion of the <i>ldh</i> , <i>ldh</i> , <i>pfl</i> , and <i>mdh</i> genes encoding D- and L-lactate dehydrogenase, pyruvate formate lyase, and malate dehydrogenase, respectively.	Engineering <i>V. natriegens</i> for the anaerobic biosynthesis of alanine	[141]

Table 2 (continued)

Bacterial species or strains	Vector ^a	Methods of manipulation	Characteristics of the introduced DNA	Expressed genes	Technological interest	Ref.
<i>Vibrio vulnificus</i> ; <i>Vibrio parahaemolyticus</i> ; <i>V. cholerae</i>	pVv3 (<i>lacZ</i> ; <i>Km^r</i>)	Electroporation	Circular plasmid	- Gfp; - <i>vvhBA</i> hemolysin operon	Establishing efficient electroporation procedure for <i>Vibrio</i> through the pVv3 vector	[85]
<i>Vibrio</i> sp.	Suicide T-Vectors pLP11-T and pLP12-T (derivative of pSW23T; <i>Cm^r</i> ; <i>vmi480</i>)	Conjugation	Circular plasmid	<i>vmi480</i>	Developing a genetic manipulation system based on a counterselectable marker for <i>Vibrio</i> spp.	[127]
<i>Vibrio</i> sp.	- pKV111-GFP (Ori p15A; <i>Em^r</i> ; <i>Cm^r</i>) - Helper plasmid pEVS104 (ori R6K; <i>Em^r</i> ; <i>Cm^r</i>)	Conjugation with <i>E. coli</i> CC118 λ pir	Circular plasmid	Gfp	Developing a simple system for tagging wild-type marine vibrios with GFP	[125]

^a Characteristics of vectors are indicated in brackets; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tet^r, tetracycline resistance; Gm^r, gentamycin resistance; Em^r, erythromycin resistance, MCS; multiple cloning sites.

V. fischeri and *V. cholerae*. In *V. fischeri*, both the TfoY and TfoX proteins contribute to competence acquisition but with a different function, whereas in *V. cholerae*, they do not contribute directly to the natural competence [60]. Indeed, in *V. cholerae*, TfoY is more involved in the activation of type VI secretion system (T6SS), which is responsible for interbacterial killing and intoxication of eukaryotes. The T6SS system enhances HGT, thus contributing to the release of naked genomic DNA from prey cells and its acquisition by the predator cells [53]. Moreover, the form of chitin capable of inducing cell competence differs among species: in *V. fischeri*, only the oligosaccharidic chitin can induce competence, whereas all forms of chitin induce competence in *V. cholerae*, *V. vulnificus* [23,59,61], and *V. parahaemolyticus* [58]. In *V. vulnificus*, the disaccharide GlcNAc2 also induces competence, but not its monomer GlcNAc [62].

Bacteria use restriction endonucleases to protect their DNA from bacteriophage infection, excess mutation, and genome rearrangements induced by exogenous DNA. These systems decrease the efficiency of natural transformation and are peculiar to each species. *V. cholerae* has two extracellular nucleases, Dns and Xtr, which degrades the exogenous DNA, thus preventing its internalization. The expression of the Dns nuclease is induced at low cellular density by the quorum-sensing regulator HapR, and the expression of the nuclease is repressed at high cell density [55]. The authors suggested that the Dns nuclease, which is active at low cell density, favors a rapid cell growth, thereby supplying the cells with nucleotides. Conversely, when the population reaches a high cell density, the uptake of exogenous DNA represents an adaptation strategy through genome diversification [55].

The natural acquisition of plasmid DNA has also been reported in *Vibrio* by cell-mediated contact, still utilizing chitin induction for competence [63]. The authors showed that *Vibrio* strains were capable of taking up nonconjugative plasmids from *Escherichia coli* donor cells. The transfer was not by conjugation and occurred with live or dead donor cells. Nevertheless, the plasmid acquisition necessitated cell–cell contact, and the process did not occur when the donor and the recipient cells were separated by a 0.2- μ m filter [63]. The same system has been also described in soil bacteria by Wang et al. [30].

The natural transformation of *Vibrio* sp. is considered as a driver for its ecological diversification. It is likely that the virulence of *V. cholerae* is the result of several HGT events, which could explain the evolution of this marine bacterium into a major human pathogen [64]. Similarly, the transfer of the genes that encode for the capsular polysaccharide (CPS), which is an important factor of virulence, occurred through HGT in *V. vulnificus*, thus providing an explanation of CPS loci diversity and evolution in marine bacteria [65]. The genome analysis of two *V. fischeri* strains also demonstrated that this species underwent HGT events also by conjugation and transduction in addition to transformation [66,67].

Thus far, few studies have utilized the natural transformation as a laboratory method for the genetic manipulation of marine bacterial strains (Table 1 and Table 2) [39,60]. For instance, Pollack-Berti et al. [60] used a simple and efficient protocol to naturally transform *V. fischeri* with exogenous DNA. Specifically, the strain had been grown to the mid-log phase in minimal medium supplemented with soluble chitin oligosaccharides as the carbon source (e.g., chitohexaose). Natural transformation of this strain has been achieved at high frequencies, thus providing a useful tool for the genetic manipulation of the species.

3. Artificial competence of marine bacteria

3.1. Chemical transformation of marine bacteria

Artificial transformation was applied for the first time by Mandel and Higa [68]. The *E. coli* cells were treated with a solution of CaCl₂, and this made the cells competent for the uptake of exogenous DNA. Subsequently, Cohen et al. [69] demonstrated the process of bacterial transformation; the exposure of cold suspensions of bacterial cells to DNA and the subsequent application of thermal shock at 42°C created pores in the cellular membrane, thereby promoting the DNA uptake (Fig. 1).

Attempts to transform marine bacteria by chemical procedures have been reported, but not always with good results (Table 1). For instance, Marcus et al. [70] were not successful in transforming *V. cholerae* strains by using the osmotic shock protocol. Attempts have been performed by suspending the cell pellets in cold hypotonic MgCl₂ and CaCl₂ solutions and by adding the plasmid pBR322 (80 ng/ml) to the pellet-containing solution; this is followed by the heat shock treatment at 42°C and the selection of transformant colonies on Brain Heart Infusion agar plates [70]. On the other hand, the use of a DNase-negative mutant of *V. cholerae* showed positive results, suggesting that extracellular DNase was a major barrier for the transformation [70]. The authors successfully transformed wild-type and DNase-negative *V. cholerae* strains by electroporation, thus evaluating the influence of different parameters (e.g., field strength, plasmid size, and electrolyte composition of the buffer) and emphasizing that host restriction systems were not a significant barrier of transformation, as observed in the osmotic shock attempts [70]. Electroporation was used as a successful method for the transformation of *V. parahaemolyticus*, *V. alginolyticus*, and *V. cholerae* [71].

Chemical transformation approaches were unsuccessful also for other genera. Piekarski et al. [72] attempted to transform 12 *Roseobacter* sp. strains using the standard transformation procedure of *E. coli*. The experiment was not successful, but, conversely, the electroporation procedure produced positive results by using a protocol that included, at least, five washings with glycerol to

effectively remove the salts from the bacterial cultures during the preparation of the electrocompetent cells. Recently, *Vibrio natriegens* has been manipulated by both chemical transformation and electroporation [73]. The transformation was successful by both methods, thereby showing efficiencies of 10^5 – 10^6 CFU/ μg of plasmid DNA (pACYC184) and 10^6 – 10^7 CFU μg^{-1} of plasmid DNA (pACYC184) by chemical transformation and electroporation, respectively [73]. However, chemical transformation produced successful results with a mutant strain that carried a deletion in the chromosomal Dns endonuclease. These results highlight that more efforts should be directed toward the investigation of the transformability of marine bacteria in relation to the host restriction system; this can help in analyzing efficient ways to impair the bacterial restriction modification defense response. Another important factor to be considered is the presence of salts, which are required for the cultivation of the majority of the marine strains, but these salts could affect the subsequent steps of the transformation protocol, particularly in the case of electroporation (as explained in the following paragraphs).

3.2. Electroporation of marine bacteria

The breakthrough of transformation procedures has been made with the discovery that an electric pulse of around 5–10 kV/cm applied to cells could induce cell membrane permeability by transient pore formation, thereby promoting cell uptake of DNA and allowing transformation of a large range of bacteria [74,75]. Currently, exogenous DNA is broadly inserted into bacterial cells by electroporation, which includes few steps. First, cells are made competent to acquire exogenous molecules by exposing the cells to several washings in cold, low ionic strength buffers; this allows the elimination of remaining ions and substrates/metabolites from the growth medium to stabilize cell membranes and to facilitate DNA binding. Subsequently, the cells are exposed to an electric field in the presence of DNA, and after the pulse is applied, the mixture is incubated under appropriate conditions for bacterial repair. This incubation step allows cell repair, but not cell growth, because the cells are not yet under the pressure of the selective markers present in the acquired DNA (Fig. 1). Conventional protocols for *E. coli* electroporation apply 1-hour incubation in a nutrient-rich medium for cell repair, whereas longer incubation time would be required for strains that have longer duplication time [76,77]. Finally, putative transformant colonies are selected on agarized media by utilizing the advantage of the proper selection markers. The review by Aune et al. [32] describes the different procedures used in artificial transformation and the range of bacteria that can be transformed; nevertheless, no reference to marine bacteria is present.

The mechanism of electroporation is not clearly understood [78,79]. The electroporation efficiency is strain dependent, but additional factors can influence the experimental success, e.g., growth conditions, pulse applied, and type of exogenous DNA [80]. A range of marine strains that belong to different genera have been successfully transformed by electroporation, e.g., *Roseobacter*, *Vibrio*, *Pseudoalteromonas*, *Caulobacter*, *Cyanobacteria*, and *Halomonas* [41,72,73,77,81,82,83,84] (Table 1 and Table 2). Particularly, the genetic manipulation of some pathogenic marine bacteria has been carried out to further investigate the bacterial virulence mechanisms. For instance, the shuttle vector PVv3 has been recently constructed from the small naturally occurring plasmid pVN-0126 to study gene expression in *V. vulnificus* [85]. This plasmid could be easily introduced into other *Vibrio* strains, i.e., *V. parahaemolyticus* and *V. cholerae* by electroporation [85]. By using this plasmid, the transformation efficiency has been optimized for the *V. vulnificus* strain VN-0101 according to different parameters, e.g., growth phase, DNA amount, electric field strength, pH, and sucrose concentration of the electroporation buffer, and it was found that

higher electroporation efficiencies (up to 2×10^6 per μg of DNA) could be obtained with 10–25 ng of plasmid DNA [85].

Although electroporation is a well-established method that is successful on a broad range of species, failure of transformation has been recorded. Many factors could affect electroporation efficiency, e.g., cell concentration, which displays considerable impact on transformation [86], or temperature, which could affect the physical properties of the cell membrane by changing lipid composition and membrane fluidity [87]. In the following paragraphs, we emphasize the main factors that can affect electroporation efficiency.

3.2.1. Effect of growth medium and buffer composition

The composition of the medium used to propagate cells before electroporation can affect the physiological state of the cell membrane [32]. It has been reported that the addition of membrane-weakening agents to the bacterial growth medium, such as glycine – which is the most commonly used weakening agent – can enhance the efficiency of bacterial transformation because they act as destabilizers of the peptidoglycan crosslinking [77,88]. However, the concentration of these agents needs to be carefully established because high concentrations can be toxic, thus hampering the viability of cells [32]. The salinity of the medium is particularly relevant for the marine bacteria, which usually require a growth medium supplemented with NaCl to maintain a high ionic strength environment, thereby mimicking their original habitat [82]. However, the presence of salts is one of the most important factors impeding the electroporation process [33]. Conversely, the addition of cations during the washing steps has been reported to have positive effects on the transformation success: on one hand, cations act as stabilizers of the membrane, and on the other hand, they can bind to DNA, thus minimizing the charge repulsion effect between the membrane and the DNA itself [89]. In *E. coli*, the presence of cations such as Mg^{2+} in the growth medium and buffer solution has been reported to weaken the bacterial membrane, thereby increasing the transformation efficiency [90,91]. Nevertheless, the use of a buffer that lacked Mg^{2+} increased the transformation efficiency in *V. parahaemolyticus*; this is because of the reason that Mg^{2+} ions are required by DNases to digest DNA, thus affecting transformation efficiency by putatively decreasing the amount of donor DNA in the cell [92]. Hence, buffers that lack Mg^{2+} are recommended for bacteria with a functional nuclease restriction system [92]. Furthermore, the use of high concentration of Mg^{2+} (10 mM) can result in an arcing effect when electrical field is applied. The concentration of salt also affects the activity of restriction enzymes, as in the case of *V. cholerae* for which Dns endonuclease is more active in presence of salt (at 175 mM NaCl at pH 7.5–8.0) [48]. A protocol based on the evaluation of different electroporation parameters has been developed for the marine strain *Bacillus marinus* B-9987 that showed an increased transformation efficiency by using glycine betaine (7.5%) as an osmoprotectant, 1 mmol L^{-1} HEPES and 2 mmol L^{-1} MgCl_2 in the electroporation medium, and unmethylated plasmid as the transforming DNA (see Section 3.2.4) and by applying field strength of 20 kV cm^{-1} (Liu et al. 2014). The developed protocol has been then used to successfully transform other strains such as the marine isolate *Bacillus licheniformis* EI-34-6 [77].

Other organic compounds have been demonstrated to have a beneficial effect on the transformation efficiency. Gilchrist and Smit [82] reported that treatment of the cells of cyanobacteria with EDTA-containing buffer disrupts the integrity of the paracrystalline surface (S) layer that surrounds the cells, thus improving the transformation up to 50% rather than without using EDTA. Among a range of different buffers (phosphate salts, HEPES, Tris-HCl, and glycerol), sucrose has been identified as the best stabilizer of cell membrane for *Vibrio* [41], whereas the addition of 10–15% of ethanol to the electroporation buffer allowed the transformation of *Oenococcus oeni*, which was previously reported as a nontransformable strain [93], probably by affecting membrane fluidity [94]. The addition

of mercaptoethanol during the bacterial growth and in the transformation mixture before electroporation increased the transformation of *E. coli* strains [95].

3.2.2. Effect of field strength

In general, bacterial strains require high voltage to be efficiently transformed. This is the case of the marine *Caulobacter* CB2A strain, for which optimum transformation has been achieved with a field strength of 12.5 kV cm^{-1} (25 μF capacitance, 4.2 ms time constant, and 400 Ω resistance) [82]. However, field strength of 6.25 kV cm^{-1} but with higher capacitance (125–960 μF) significantly reduced the survival rate of cells [82]. Moreover, the marine fish pathogens *Vibrio anguillarum* and *Pasteurella piscicida* required optimal voltage strength of 12.5 kV cm^{-1} and time constant of 5 ms to be transformed efficiently with the three plasmids pSU2718, pCML, and pEV3 of molecular sizes 2.6, 5, and 13.7 kb, respectively [96].

High-voltage electroporation has been reported to be deleterious in the case of other marine strains. In a recent study, Harris et al. [83] showed a tenfold increase in the transformation efficiency of *Halomonas* sp. O1 at a voltage from 7.5 kV cm^{-1} (10^3 CFUs μg^{-1} of DNA) to 10.5 kV cm^{-1} (10^4 CFUs μg^{-1} of DNA), followed by a slight decrease in efficiency at a voltage of more than 10.5 kV cm^{-1} . A voltage of 2 kV cm^{-1} (4000 μF) was regarded as the best field strength in the case of *Synechococcus* sp. CC9311 transformed with the voltage range of 1–4 kV cm^{-1} [84]. With regard to the transformation of different *Roseobacter* strains, Piekarski et al. [72] reported that 12.5 kV cm^{-1} (25 μF , 200 Ω , and 0.2-cm cuvettes) was the optimum voltage among the different pulse intensities tested (7.5–15 kV cm^{-1}). The voltage strength has also been reported as a crucial value for the transformation of *Fischerella muscicola* PCC 7414, which was transformed with plasmid DNA at 1.6 kV cm^{-1} , 600 Ω , and 15 ms, among a range of values varying from 0.6 to 1.9 kV cm^{-1} , from 5 to 15 ms, and from 200 to 600 Ω [97].

3.2.3. Effect of plasmid size, topology, concentration, and codon usage pattern

In general, transformation efficiency increases proportionally to DNA concentrations until a saturation level, which seems to be specific to each species, without negative effects when higher DNA concentrations are applied [98,99]. Indeed, Piekarski et al. [82] reported that the number of transformant cells obtained is proportional to the amount of plasmid DNA added during the electroporation of *Caulobacter* strains. It has also been found that the efficiency of transformation decreases with the increase in plasmid size [80,100,101]. However, some exceptions have also been reported for *B. subtilis*, for which the transformation efficiency was not affected by the plasmid size [102].

DNA topology is another important determinant that has been demonstrated to influence electroporation efficiency in both *B. subtilis* and *E. coli* [102,103]. According to Xie and Tsong [103], the translocation of DNA across the membrane in the *E. coli* strain JM105 was not affected by the topology of the DNA when tested in experiments with circular supercoiled (scDNA), circular relaxed (crDNA), and linearized (lnDNA) forms of the plasmid pBR322. However, the transformation efficiency with lnDNA was lower than those with crDNA and scDNA because of the instability of lnDNA in the host cell, where it was rapidly degraded by the host enzymatic system. Similarly, Ohse et al. [102] demonstrated that the electrotransformation of *B. subtilis* ISW1214 with the three aforementioned topological isomers of the plasmids pUB110 (4.5 kbp) and pBDR331T (12.6 kbp) produced positive results for the scDNA and crDNA forms of both plasmids but a negative result for lnDNA. This could mean that either lnDNA is unstable in the cytoplasm of *B. subtilis* or that it could not be converted to the crDNA form in the host cell, which is an essential step for the replication of the plasmid in the cell.

To our knowledge, the effects of DNA topology on transformation efficiency have never been tested on marine isolates. We can hypothesize, however, that this factor could affect the transformability of marine strains, as demonstrated for the model strains *B. subtilis* and *E. coli*; thus, DNA topology should be considered in the array of methods for the manipulation of novel isolates.

Once the exogenous DNA is internalized by any given bacterium, its expression could be influenced by the codon usage bias of the host. Similar to other bacterial species, marine bacteria have synonymous codons that are not used with the same frequency, and this creates what has been defined as codon usage bias. This phenomenon results from the equilibrium of natural selection, mutation, and genetic drift [104]. Few studies have been carried out using marine bacteria with an aim to investigate their codon usage, mainly considering the species *V. cholerae* [104,105,106]. This bacterium shows an atypical high codon usage bias for genes with low potential expression, thus hypothesizing that nonoptimal codons are used to maintain low cellular concentrations of the encoded proteins [104]. Codon optimization could be an important factor to be considered when exogenous genes are inserted into other hosts by heterologous expression. To date, this has been demonstrated in the nonmarine strain *Frankia* sp., in which the use of a codon-optimized gene, which encodes for an antibiotic resistant determinant, improved the efficiency of bacterial transformation [107].

3.2.4. Effect of restriction enzyme systems

The majority of the bacterial cells own specific restriction modification systems that prevent the excess of variability introduced into genomes by recombination with exogenous DNA or by lytic phage infection. These systems defend bacteria from the entrance of DNA mediated by viruses or contact with other cells, thus degrading the phage-derived or exogenous DNA at specific sites. Therefore, if the exogenous DNA is modified at these specific sites, it cannot be recognized by the restriction systems, which selectively digest exogenous DNA by differentiating it from host endogenous DNA depending on the host-specific DNA methylation pattern [108]. Consequently, defense systems of bacteria are responsible for preventing the transformation occurrence. However, an exogenous DNA with a methylation pattern that imitates the host-specific methylation pattern (host-mimicking DNA) could be incorporated into the recipient [109]. Yasui et al. [110] developed an efficient method to increase the transformation efficiency of genome-sequenced bacteria using “Plasmid Artificial Modification” (PAM), in which the plasmid vector to be inserted into the bacterial host is premethylated in *E. coli* according to the host-specific restriction system. Wallace and Breaker [111] adopted this method and constructed a specific PAM for a soil-inhabiting strain of *Bacillus halodurans*, thereby increasing the transformation efficiency by 10- to 1000-fold in the recipient bacterium.

In addition, the presence of restriction endonuclease enzymes in Cyanobacteria putatively impairs their transformation efficiency. These bacteria are considered to be easily manipulated because a range of strains that belong to this phylum have been successfully transformed by natural transformation and electroporation [112,113]. However, some Cyanobacteria species remain recalcitrant to incorporate exogenous DNA, and their transformation has been achieved only by using premethylated DNA [97,109]. Another strategy to bypass the restriction enzyme barrier was developed in marine *Vibrio* strains by Kawagishi et al. [114] in 1994; they showed that exposure of *Vibrio* cells to an osmotic shock before electroporation enhanced the membrane permeability, thereby allowing periplasmic DNase excretion.

Depending on the type of the bacterial restriction systems, unmethylated DNA could also be successfully used in transformation experiments. For instance, the transformation efficiency in *B. marinus* B-9987 using unmethylated DNA plasmid pHT3101 was significantly

increased by around 300- to 1000-fold compared to the use of the methylated DNA plasmid pHT3101. This could be due to the fact that *B. marinus* B-9987 encodes restriction enzymes against Dam-methylated and/or Dcm-methylated DNA, but it has no effects on the unmethylated DNA [77].

Both natural and artificial transformations can be ineffective depending on the bacterial strain of interest and the transforming DNA. For instance, in *Vibrio splendidus* LGP32, attempts to transfer plasmids using electroporation failed, but when the authors transferred a novel RP4-based suicide vector, they succeeded in obtaining several mutants including a strain with the deleted metalloprotease gene *vsm*, which was then used to decipher the virulence property of the gene [115].

The genetic manipulation of the typical marine genus *Pseudoalteromonas* is hampered by the salt-dependent growth of its species because salt acts as a barrier in preventing the binding of DNA to the cell surface. Moreover, the abundant distribution of restriction modification systems in the genome decreases DNA internalization efficiency, and the presence of multiple resistance genes and drug efflux pumps allows the cells to overcome the antibiotic pressure that hinders the counter-selection of transformants [33]. Conflicting results have been reported for the manipulation of *Pseudoalteromonas* strains. The study published by Zhao et al. [116] showed that the nonmobilizable plasmid vector pWD2 could efficiently transform its original host strain through electroporation. Conversely, Wang et al. [33] failed to electroplate the vector pWD2 in other *Pseudoalteromonas* strains but could transfer its derivative pWD2-oriT by conjugation with *E. coli* as the donor cells.

4. Conjugation in marine strains

The horizontal transfer of genetic material among marine bacteria occurs naturally by conjugation, which is mediated by cell-to-cell contact between the phylogenetically closely and distantly related strains; it involves the transfer of mobile elements such as plasmids, transposons, and integrons [73,117,118,119]. The bacterial conjugation machinery relies on the origin of transfer, also known as oriT sequence, and *tra* genes, which encode the relaxase proteins, mating pair formation (MPF) complex, and type IV coupling protein. The transfer apparatus is developed particularly in gram-negative bacteria [120]. Recently, evidence obtained with *Dinoroseobacter shibae* and *Phaeobacter inhibens* suggested that conjugation in *Roseobacter* is controlled by quorum-sensing mechanisms [121]. In some strains of the extremophile *Thermus thermophilus*, a particular transformation-dependent conjugation has been described and termed “transjugation” [122]. The process is bidirectional, has higher efficiency than transformation, and requires a cell-to-cell contact. The authors proposed a mechanism that involves two steps; according to the mechanism, the donor cell pushes out the DNA and the recipient cell takes up through the natural competence apparatus [122].

Functions necessary for transfer by conjugation can be provided on one individual plasmid (donor strain), or be present on more plasmids, thus involving a helper strain in addition to donor(s) strain. If one plasmid encodes the complete conjugation machinery, then biparental mating occurs [123]. Otherwise, if a helper strain is necessary, with a helper plasmid encoding the transfer functions to move the mobilizable plasmid from the donor to the recipient strain, then the process is defined as triparental mating [119]. In four-parental mating (a variation of the triparental mating), a helper strain allows the transfer of two mobilizable plasmids from two donor strains into the recipient strain [123,124]. The application of conjugation protocols in laboratory is a time-consuming procedure that also requires a specific step to counter-select the donor strains from the transconjugant strains from the conjugation mixture (Table 1; Fig. 1). However, conjugation is the most efficient way to

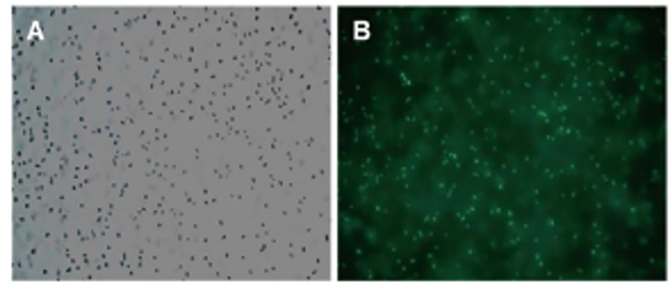


Fig. 2. Phase-contrast (a) and fluorescence (b) microscopy images of the marine strain *Halomonas axialensis* M10 chromosomally tagged with a green fluorescent protein (Gfp) using the mini-Tn7 transposon system delivered by conjugation [124]. The strain was isolated from the seawater-brine interface of the deep hypersaline anoxic basin (DHAB), Urania, located in the Eastern Mediterranean Sea [7].

genetically manipulate strains that are recalcitrant to be transformed by electroporation and chemical methods (Fig. 2).

A main issue in the setup of a conjugation protocol is the selection of the donor cells from the transconjugant cells after mating because the transfer of the mobile element confers to the donor and the recipient the same encoded trait. Many selection systems have been developed with this aim. Sawabe et al. [125] tagged 39 *Vibrio* sp. strains with a green fluorescent protein (GFP) cassette by biparental mating with *E. coli*; the conjugation mixture was incubated on the ZoBell 2216E agar, which contains 0.5% sodium alginate medium at 15°C, thus allowing the growth of both strains [124]. Then, transconjugant selection was achieved on a different medium specific for the selective growth of *Vibrio*. Travers et al. [126] developed another system to tag the fish pathogen *Vibrio harveyi* with GFP cassettes, and following the conjugation process, analyses were performed by flow cytometry and epifluorescence microscopy. The conjugative process was performed by triparental mating, which involves the donor strain *E. coli* DH5 α , carrying the GFP gene on the plasmid pSV102; the helper strain *E. coli* CC118; and the recipient *V. harveyi* strain ORM4. Green fluorescence of the recipient cells was verified by epifluorescence microscopy, and *Vibrio* cells were distinguished from the *E. coli* donor cells under a microscope by the presence of flagella and the ability to swim [126]. Rare conjugation events nevertheless need to be positively selected on the basis of differential growth conditions, or physically separated from by flow cytometry-based cell sorting.

Recently, Luo et al. [127] developed an efficient system of gene deletion in *Vibrio* sp. by constructing the two suicide plasmids pLP11 and pLP12 (derived from the plasmid pSV23T), which carry the chloramphenicol antibiotic resistant gene and a novel counter-selectable marker *vmi480* and are able to form a toxin-antitoxin complex with the gene *vmi470*. The authors proceeded to obtain knockout mutants by constructing an efficient conjugation system in which *E. coli* B1263 (donor of the suicide vectors) cannot grow on thymidine or diaminopimelic acid (DAP); therefore, there was no need for a recipient selection marker. In particular, species such as *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*, and *V. alginolyticus* have been considered. The sole expression of *vmi480* is lethal to the cells, but the lethal effect can be eliminated when *vmi480* and *vmi470* are co-expressed in the same vector. Therefore, *vmi480* is considered as an effective counter-selectable gene for *Vibrio* [127]. Although *sacB*, which is a well-known counter-selection marker in gram-negative bacteria, has also been used in *Vibrio* sp., the toxicity of *sacB* is susceptible to the presence of NaCl (required in the growth medium for the majority of the marine bacteria) in the selective medium. Therefore, the inadequate toxicity of *sacB* can decrease the selection efficiency in many marine bacteria.

Genetic manipulation systems for *Pseudoalteromonas* strains have been developed by Yu et al. [128] and Wang et al. [33].

Pseudoalteromonas sp. are ubiquitous bacteria in marine environment and play important biological and ecological roles in deep-sea sediment ecosystem [128]. To date, more than 50 *Pseudoalteromonas* genomes have been sequenced. *Pseudoalteromonas* cannot be efficiently transformed by electroporation because of its restriction modification systems that prevent the electroporation of double-stranded DNA [33]. Thus, Yu et al. [128] constructed a conjugation system to tag the deep-sea psychrophilic bacterium *Pseudoalteromonas* sp. SM9913, by using *E. coli* ET12567 as the donor of the vector pOrit-4Em, thus obtaining an efficiency of 1.8×10^{-3} . Transconjugants were selected from the *E. coli* donor at growth temperature of 20°C, which resulted restrictive for *E. coli* [128]. Moreover, a suicide vector has been successfully constructed for the same strain, using the pOrit-4Em vector and the *sacB* gene as the counter-selection marker to knockout the *epsT* gene that encodes the UDP-glucose lipid carrier transferase. Wang et al. [33] selected *Pseudoalteromonas haloplanktis* TAC125 conjugants from *E. coli* donor cells by growing the conjugation mixture at 4°C, which is not suitable for *E. coli* growth. With this system, nevertheless, knockout mutants of genes involved in the bacterial cold adaptation cannot be obtained because their absence affects the transconjugant counter-selection [33].

In addition to the above-mentioned traits, the use of spontaneous antibiotic-resistant mutants of the recipient strains is commonly utilized for the counter-selection of transconjugants against *E. coli* donor cells [129]. Actually, such mutations may also cause indirect pleiotropic effects that might influence the general physiology of the target strain, which consequently affects the mutant growth behavior [130]. Moreover, the use of antibiotics as selection markers could affect the conjugation efficiency, particularly in the case of halophilic marine bacteria owing to their requirements of saline media because several antibiotics including tetracycline and gentamicin have a decreased activity in the presence of high salt concentrations [131]. Other factors could also affect antibiotic susceptibility. For example, many *Pseudoalteromonas* strains are sensitive to chloramphenicol and erythromycin, which could be hence utilized for transconjugant counter-selection. However, false-positive colonies may appear when chloramphenicol is used for the selection of transconjugants; therefore, erythromycin is recommended the most as an effective selective marker for these strains, although the basic mechanism of this behavior remains unclear [33].

For the above-mentioned reasons, auxotrophic donor strains are better adopted. Wang et al. [33] developed a universal efficient conjugation system for *Pseudoalteromonas* strains by using shuttle vectors and suicide vectors. The system was based on RP4 conjugation machinery in *E. coli* WM3064, which is auxotrophic for DAP. Conjugation efficiency for nine *Pseudoalteromonas* strains was measured as 10^{-6} – 10^{-3} transconjugants per recipient cell; the selection of the recipient transconjugants was performed on modified LB mating medium (MLB) devoid of DAP [33]. Furthermore, a conjugative system was constructed for *Roseobacter* clade using a plasmid that encodes, as reporter gene, the flavin mononucleotide-based fluorescent protein (FbFP protein) and a donor *E. coli* strain, auxotrophic for aminolevulinic acid (ALA) [72]. A genetic system was also constructed for *Marinobacter adhaerens* HP15 by using the two plasmids pBBR1MCS and pSUP106, through biparental and triparental mating, with *E. coli* ST18 as the donor and *E. coli* HB101 as the helper. The selection of the transconjugants was performed by marker selection and utilizing the donor ALA-auxotrophic feature [132].

In case of Cyanobacteria, a genetic manipulation system has been successfully achieved for *Prochlorococcus* MIT9313 by transferring RSF1010-derived plasmids, i.e., RL153, pRL153-GFP, and RL27, in conjugation experiments with *E. coli* donors [133]. Particularly, the plasmid RL27 contained a mini-Tn5 transposon, thus showing the possibility to randomly inactivate genes by transposon mutagenesis. The selection of transconjugants has been performed by growing the conjugation mixture on a medium poor in the appropriate antibiotic.

Because the medium was not efficient to inhibit the growth of all *E. coli* donors, remaining donors have been eliminated by using *E. coli* phage T7. Importantly, transconjugant colonies did not appear on the selective medium after mating; they required at least one step of culture in liquid medium before isolation [133]. An improvement step for conjugation in Cyanobacteria has been mentioned by Stucken et al. [97] who showed that washing cells with NaCl (1 M) before conjugation contributed to an increase in the efficiency by weakening the thick cell wall, rich in exopolysaccharides.

The conjugation efficiency could also be affected by the ratio of the donor to recipient cells, which varies upon strains. The best conjugation efficiency for *Roseobacter* has been obtained at *E. coli* donor-to-*Roseobacter* recipient ratios of 5:1 and 10:1 [72]. In case of *Pseudoalteromonas* sp. SM9913, the transfer efficiency is optimal at a donor-to-recipient ratio of 100:1 [128].

5. Genome engineering in marine bacteria

Bacterial transformation and conjugation are useful to mediate the delivery of genetic determinants targeting the bacterial chromosomal DNA by utilizing homologous recombination and transposition-based systems (Fig. 2). In recent years, several techniques that enable bacterial genome engineering and editing, such as knockout, knockin, and insertional mutations, have been developed. For instance, clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (CRISPR-Cas 9 systems) allowed convenient and simple editing of both bacterial and eukaryotic genomes [134,135]. This system has been applied to *E. coli* [136] and *Streptomyces* strains [137] among others. With regard to marine bacteria, the CRISPR/Cas 9 system in conjunction with the lambda Red recombinase system has been used to knockin the fatty acid metabolic genes from a lipid-rich marine bacterium, *Shewanella frigidimarina*, to *E. coli* to investigate the genes related to fatty acid biosynthesis [138].

To leverage genomic information and characterize gene functions in marine bacteria, more genetic manipulation systems are required and new tools have been recently developed. Multiplex genome engineering techniques by natural transformation (MuGENT) have recently been developed for the marine *V. cholerae* and *V. natriegens* [139,140,141]. Converse to the introduction of single mutations in the bacterial genome, MuGENT allows an efficient and rapid genome editing by simultaneously generating multiple mutations. The approach is based on the ability of each natural competent strain to internalize multiple DNA molecules [139]. With the application of the MuGENT approach, cells are incubated with two types of InDNA: the first is a selected product that incorporates an antibiotic resistance marker into the genome and the second is an unselected product that introduces scarless genome edits at one or more loci. In *V. natriegens* (made naturally competent by the ectopic expression of the endogenous *tfxX* gene [140]) MuGENT proceeded to target the nine genes involved in poly-*B*-hydroxybutyrate production by regulating the expression or inactivating genes that affect its synthesis [140].

6. Conclusions

In the last decades, HGT processes among bacteria [23] and between bacteria and their hosts (such as plants [142,143] and animals [35]) have been a focal point of microbial molecular biology and genetic studies. Genetic manipulation methods have been nevertheless applied to marine bacteria to certain, but limited, extent [34,127]. Presently, this has an impact, especially in the wake of the improvements of isolation and cultivation procedures, on utilizing the biotechnological potential of the novel isolates. Different genetic manipulation systems including chemical transformation, electroporation, and conjugation have been proven to be useful to manipulate marine bacteria [72,132,144]. It is a challenging task to predict which method would be the most effective for any given bacterium. In general, electroporation and conjugation

are regarded as the most efficient methods to genetically manipulate marine strains [33,72,77,82]. Nevertheless, the success of genetic manipulation relies on different variables that must be examined in depth and that need to be optimized for each single strain/species (Table 1). These variables include both technical characteristics of the methodologies and bacterial growth features. The use of NaCl for the growth of marine bacteria is the main obstacle that affects the manipulation of the bacteria. In natural competence, electroporation, and chemical transformation protocols, the presence of NaCl in the growth medium is fastidious and prevents, to some extent, the DNA entry into the cytoplasm. Therefore, during the preparation of competent cells, specific washings of the bacterial culture are necessary to eliminate the remaining ions from the culture medium. Furthermore, the presence of salts in the selective medium can also affect the susceptibility of bacteria to antibiotics that are used as selective markers of transformants or transconjugants. For instance, the efficacy of tetracycline and gentamycin can be affected by high salt concentration in the marine broth medium; moreover, kanamycin is chelated by Cu^{2+} , whereas tetracycline is chelated by divalent cations such as Mg^{2+} and Ca^{2+} . The formation of chelates may not have a significant impact on the antibiotic stability, but it can decrease its bioavailability [72].

In the construction of suicide vectors, which are important systems in gene deletion, *sacB* is one of the most common counter-selection genes for gram-negative bacteria. The induction of *sacB* requires the presence of sucrose in the selection medium, but its toxicity is affected by the presence of NaCl, thus hampering its use as counter-selection marker for marine bacteria. For this reason, Travers et al. [126] proposed a successful suicide vector that is induced by L-arabinose. This system was designed to be used in the gene disruption of *Vibrio* strains, but it remains to be tested on other marine gram-negative bacteria.

Optimization of NaCl concentrations could also aid to overcome another important barrier for artificial transformation represented by the restriction modification defense systems. In *V. cholerae*, the Dns endonuclease is more active in the presence of salt, i.e., with 175 mM NaCl [48]. Therefore, the use of a growth medium with a low content of salts, but still capable of sustaining the bacterial growth, could be validated for genetic manipulation experiments of marine bacteria.

Although there are several efficient genetic manipulation methods, it is very difficult to design a universal strategy to efficiently transform any bacterium with exogenous DNA. Hence, further research in this field is required, with an aim to optimize protocols for specific bacterial species or groups. The procedures of testing different parameters such as DNA concentration and size, electric field voltage, different buffers and their composition, bacterial cell density, temperature, and incubation time of the transformation/conjugation mixture will be time consuming, but they will enable an in-depth investigation of both physiological properties and biotechnological potential of the marine bacteria, which have been recognized as an untapped source of genetic and functional diversity.

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Conflict of interest

The authors declare no conflict of interest.

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