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Review Article

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Historical Perspectives on the Bacterium *Vibrio natriegens* and its Potential to Revolutionize Bioengineering

Abstract

Background: *Vibrio natriegens* is an aquatic bacterium that has the fastest doubling time of any currently known organism at approximate 9.8 min. This review delves into the early categorization of *V. natriegens*, its phylogeny and physiology, and the efforts aimed at studying its potential to enhance both micro- and macro-scale biotechnology.

Methods: Twenty-eight research papers from scientific literature databases including PubMED (US National Library of Medicine), National Center for Biotechnology Information (NCBI), and the American Society for Microbiology Journals were used in this study.

Summary: Almost sixty years after the original isolate, microbiologists and bioengineers alike are expressing a renewed interest in *V. natriegens* as a possible replacement for *Escherichia coli*. Recent biotechnological efforts have been successful in developing the necessary genetic systems for such a transition. The productivity of *V. natriegens* suggests that the organism can also be used in large scale bio-refineries producing nutraceuticals and other bio-products.

The Historical Taxonomy of *Vibrio natriegens*

Discovery of the organism and classificiation as a *Pseudomonas*

William Payne first isolated *V. natriegens* from the coastal salt marshes of Sapelo Island, Georgia, USA in 1958. Together with associate R. G. Eagon, Payne studied and categorized the newly discovered bacterium at the University of Georgia in Athens. Originally listed as "marine isolate 11," *V. natriegens* was then classified in 1961 as *Pseudomonas natriegens* due to physiological observations at the time.(1,2) This marine organism is a gram-negative bacterium, the same category as *E. coli* and *Helicobacter pylori*; these bacterium contain periplasmic spaces between their outer and inner membranes.(3) Additionally, *V. natriegens* is quite small, approximately 1 μm in length, and contains a polar flagellum.

Payne and Eagon concluded that the isolated *P. natriegens* required sodium for growth and produced carbon dioxide, acetic acid, lactic acid, and pyruvate from glucose metabolism.(2) Interestingly, when they replaced sodium with other monovalent cations, such as lithium, potassium, and rubidium, in their growth medium, Payne and Eagon observed that the organism did not grow. This experiment validated that sodium was imperative for *P. natriegens* population growth.(4-6) These characteristics refined the organism's taxonomy to either the *Vibrio*, *Pseudomonas*, or *Beneckea* genera.(7, 8) These three genera belong to the Gammaproteobacteria class. Within this class, the *Vibrio* and *Beneckea* genera belong to the *Vibrionaceae* family and the *Pseudomonas* genus belongs to the *Pseudomonadacea* family. Though these families contain rod-shaped, gram-negative bacteria with polar flagella and generally reside in marine/coastal environments, *Pseudomonas* species (spp.) are aerobic while *Vibrio* and *Beneckea* spp. are facultative anaerobes.(7, 9)

At the time of *P. natriegens* classification, the phylogenies of these groups were not well defined. Early observations by Payne et al. (1961) suggested that the isolated organism could belong in any of these genera, but they chose to initially place the organism in *Pseudomonas* after observing no sensitivity to the compound 2,4-diamino-6,7-diisopropyl-pteridine, also known as 0/129.(2) 0/129 is an antimicrobial, vibriostatic agent to which only *Vibrio* bacteria are sensitive and has been used to distinguish *Vibrio* spp. from other gram-negative bacteria.

Improper classification and movement to Beneckea genus

After *V. natriegens*' initial classification into the *Pseudomonas* genus, scientists reclassified the bacterium as existing technologies were developed and improved to better understand its characteristics. In 1971, Baumann et al. proposed in "Taxonomy of marine bacteria: the genus *Beneckea*" that Payne et al.'s genus taxonification introduced in 1961 was inaccurate.(2,7) While categorizing over 145 isolates of marine bacteria which shared many general characteristics with *P. natriegens*, Baumann et al. noted that *P. natriegens* differed in physiology and behaviour from the other existing marine bacteria genera. Based on these findings, Baumann *et al.* reclassified *P. natriegens* into the novel genus *Beneckea*, where all species were gram-negative, had straight rods and polar flagella, required sodium, were unable to fix nitrogen, and had a deoxyribonucleic acid (DNA) Guanine/Cytosine content of 45-48%.(7)

In addition to physiological characteristics, Baumann et al. found that *P. natriegens* was also a facultative anaerobe, a characteristic Payne et al. had also identified. Yet, while earlier analysis showed that carbon dioxide was produced in addition to lactic, pyruvic, and acetic, Baumann et al. found that the organism produced no carbon dioxide through glucose fermentation.(2,7) *Pseudomonas* was a genus for aerobic marine proteobacteria while *Beneckea natriegens* was a gram-negative, facultative anaerobe with a rod-shape and a polar flagellum that fermented glucose without gas production.(13) The differences between Payne et al.'s and Baumann et al.'s experimental observations could have been due to several factors, including different growth conditions, experimental error, strain contamination, captive genetic adaptations, more rigorous experimental analysis, and recent technological advances.(12) These observed experimental differences supported Baumann et al.'s categorization of the bacterium into the *Beneckea* genus.

Reclassification as a *Vibrio* by Austin *et al.* in 1978.

Over the subsequent decade, more scientists grew interested in the phys-

iology of *B. natriegens* and marine proteobacteria. Microbiologists B. Austin, A. Zachary, and R. R. Colwell, revisited *B. natriegens* physiology at the University of Maryland in College Park in 1978 and realized some fundamental flaws in the previous taxonomy classification.(13) They obtained the strain Baumann et al. had been using from the ATCC organismal bank and observed that their original phenotypic analysis was accurate. Austin, Zachary, and Colwell also observed that the organism could not degrade chitin, but could use over 26 other carbon sources, including ethanol, for growth. This result was intriguing, as organisms which can use a wide variety of carbon sources generally have very complex carbon metabolic pathways. *B. natriegens*' ability to synthesize various carbon sources suggested its involvement in biogeochemical carbon cycles and its potential use in artificially biological carbon sequestration and storage initiatives.

Additionally, Austin, Zachary, and Colwell also discovered *B. natriegens* was susceptible to the vibriostatic compound 0/129, a result that contradicted Payne et al.'s original 1961 analysis.(2,13) The different observations on this vibriostatic assay by the two groups of scientists likely results from the nature of the test itself and the available technology in each time period. Researchers performing the same experiment occasionally report varied results, and in the case with *B. natriegens*'s sensitivity to 0/129, the varied mode of chemical preparation, delivery to the organism, and growth conditions all impact the detection ability. Albeit, the specific vibriostatic assay information was in neither Baumann et al.'s nor Payne et al.'s manuscripts. Nonetheless, *B. natriegens*' susceptibility to 0/129 and its phenotypic similarities with other *Vibrio* spp. prompted Austin et al. to suggest that *B. natriegens* be recategorized under the *Vibrio* genus. New evidence published by Payne in 1971 further corroborated Austin et al.'s reclassification proposal. In 1978, *B. natriegens* was renamed as *Vibrio natrigens.*(4,13)

Breaking a Scientific Record

In the early 1960s, Eagon and Payne continued their work with *Vibrio natriegens.* Eagon himself published "*Pseudomonas natriegens*, A Marine Bacteria with a Generation Time of Less than 10 Minutes," in 1962.(14) Eagon reported a record generation time of 9.8 min. for *V. natriegens* under optimal growth conditions in a brain-heart infusion broth at 37^0C with 1.5% sea salt.(14) The observed 9.8 min. generation time of *V. natriegens* was significant as the model organism at the time, *E. coli*, had a laboratory doubling time of approximately 25-30 min.(15, 16) Given how bacteria must increase their volume, replicate and proofread their DNA, synthesize proteins, and undergo cytokinesis, it is truly remarkable that *V. natriegens* can double its population in under 10 min.(17) The organism's generation time expands our limited understanding of the kinetic and thermodynamic mechanisms of cell division and organelle biogenesis.(17,18)

Genomics in Explaining Doubling Time

With recent advances in methods to quickly analyze genomes and transcriptomes, researchers are now working to understand the biochemical mechanisms for *V. natriegens*' doubling time. In 2002, Aiyar et al. attributed the bacterium's extremely fast generation time to very rapid protein synthesis rates.(19) They hypothesized that *V. natriegens* has both a higher count of ribosomes and stronger ribosomal activity mediated by powerful ribosomal RNA (rRNA) promoters and operons. Their results showed that *E. coli* produced approximately 70,000 ribosomes per cell within its 25 min. doubling time, whereas *V. natriegens* produced 115,000 ribosomes per cell within 10 min.(19) relative to a similar average cell size of about 1 micron. Through southern blotting, Aiyar et al. also identified about 13 rRNA operons in *V. natriegens* and found that the promoters of these operons were highly regulated and heavily reliant upon upstream regulators. To determine the strength of these promotors, Aiyar et al. transformed the rRNA production systems into *E. coli* and compared the protein production levels in vitro.(19)

Although knowledge of *V. natriegens* ribosomal RNA promoters and operons behaviour is a significant step towards explaining its replication rate, no genome of *V. natriegens* was available at that time. Maida et al. addressed this problem and submitted a draft genome sequence of *V. natriegens* in 2013.(20) The sequence allowed cross-referencing of *V. natriegens*' observed physical data to the reported DNA sequence and provided a comprehensive link between physiology and genetics. Maida et al. performed a physiological assessment of the bacteria under similarly ideal conditions to Eagon's 1962 experiment and noted that under strong aeration, increasing nutrient supply via mass transfer, the *V. natriegens* culture reduced its doubling time to 7 min.(14,20) With further analysis with homology search bioinformatics, the researchers also discovered 14 rRNA-encoding genes and were able to predict a total of 12 putative rRNA operons. This result aligned closely with the number of rRNA operons discovered by Aiyar et al. a decade prior.(19,20) Additionally, Maida et al.'s genomic sequencing identified closely associated rRNA promotors, information crucial for developing *V. natriegens* expression systems and genetic transformations for increasing biomass yields or productivities.

Like researchers before them, Lee *et al.* discovered 11 rRNA operons within the genome and identified 129 transfer RNA (tRNA) genes. In addition, Lee et al. provided the first complete and fully annotated genome of *V. natriegens* with improved genetic spatial organization.(22) These new findings may have implications on the doubling time of *V. natriegens* as aforementioned works have suggested that an increased number of rRNA operons and available tRNA generally results in faster doubling times.(22) More ribosomes and tRNAs increase a cell's translational capacity, enabling the bacterium to produce more functional proteins in a shorter time period. *V. natriegens* has 4 more rRNA operons and 30 more tRNA genes than *E. coli*, which could explain *V. natriegens*' impressive doubling time.(22) However, as noted by Aiyar et al., more rRNA genes cannot causally imply a faster generation time as the lack of transcriptomic information for both *E. coli* and *V. natriegens* prevents exact genomic comparisons.(19) Furthermore, limited data on translational regulation, ribosome assembly, and rRNA operon regulation has made determining mechanisms underlying *V. natriegens*' rapid doubling time difficult.

Assessing the Biotechnological Potential of V. natriegens

Since Eagon's initial categorization of *V. natriegens* in the 1950s and 60s, much of the research focus in following decades had been on the physiology explaining *V. natriegens*' growth capacity. Yet renewed interest in the organism stems mainly from a desire to apply its fast doubling time to biotechnological experiments. The use of *V. natriegens* as a surrogate organism could impact a variety of professionals, from research scientists looking for fast host-vector DNA/protein expression systems to engineers operating scaled bioreactors to mass-produce valuable bio-commodities. However, for *V. natriegens* to replace the current biological standard, *E. coli*, its full biotechnological profile needs to be developed and extensively studied, a project which many research groups have begun to undertake. Two champions in the area of *V. natriegens* biotechnology are George Church et al. at Harvard University in Cambridge, MA and members from Synthetic Genomics Inc.(SGI) in La Jolla, CA. Both research teams published papers on integrating *V. natriegens* into key biotechnological systems nearly a month apart during the summer of 2016. They presented almost identical research approaches and conclusions and highlighted the desired functionalities in a model benchtop organism.(22,23)

Researchers have also recently developed efficient transformation and recovery protocols for delivering plasmids into *V. natriegens* to yield

between 105-107 CFU/μg recombinant DNA per reaction.(22,23) *V. natriegens*' transformation efficiency is similar to that of *E. coli*; this illustrates the closeness between these two organisms as genetic surrogates. Additionally, bacterial conjugation from *E. coli* to *V. natriegens* is possible, whereby the plasmids were maintained as episomal bodies inside the latter, allowing scientists to integrate DNA from more advanced organisms and develop co-cultures to produce valuable bio-products. (23,24) It was also shown that while transformed *V. natriegens* colonies appeared after 5-6 hr, wild varieties grown on agar plates had observable colonies after only 4 hr, nearly 2.5 times faster than a similar *E. coli* system grown in rich media.(22) Weinstock et al. showed successful maintenance of antibiotic resistance cassettes (such as ampicillin, kanamycin, and chloramphenicol) within the organism and discovered that the lacI, araBAD, and λ phage pR inducible promotors function in the organism. (23) These resistance and expression systems are key components in molecular biology laboratories, and their presence in *V. natriegens* furthers its ability to compete with *E. coli* in transformation, genetic cloning, and mutant generation capabilities.

Determining optimal growth conditions, such as nutrients, temperature, and concentration, strongly impact the costs associated with efficient and beneficial energy sources. Organisms which can use a wide variety of carbon sources are inherently advantageous. In their recent publications, the Harvard and SGI teams observed that in minimal media, *V. natriegens* thrived on many carbon sources, including sucrose. Lee et al. from Harvard University reported that the organism reached the stationary growth phase after only 6-7 hr in liquid media supplemented with sucrose.(22) In comparison, *E. coli* is unable to use sucrose, a relatively inexpensive sugar, as a carbon source. Weinstock et al. from SGI were able to develop robust T7 RNA polymerase expression systems in *V. natriegens* identical to those currently found in *E. coli* and could potentially both express and recover GFP under an inducible IPTG promotor.(23) Weinstock et al. used SDS-PAGE analysis and fluorescence measurements from GFP to quantify these results.(23) Cre-Lox recombination was successfully performed for *V. natriegens* and Lee et al. were also able to develop a transposon mutagenesis system in addition to a basic CRISPRi gene regulation system.(22-24) Cre-Lox, CRISPRi, and protein expression systems are all fundamental genomic editing and analysis techniques for any organism involved in transgenic investigations, personalized medicine, mutagenesis, protein engineering, etc. The rapid development of gene and protein expression techniques and the ability of *V. natriegens* to undergo genomic engineering similar to *E. coli* establishes *V. natriegens* as a promising laboratory surrogate.

Uprooting the Status Quo

Although the baseline that technology scientists could use to substitute *V. natriegens* for other surrogate organisms in the lab is now available, it is uncertain if researchers will switch to *V. natriegens*. Even with its impressive doubling time, *V. natriegens* increases work efficiency marginally in the laboratory setting. Transformed *V. natriegens* colonies appear on solid media after approximately 5.5 hr, whereas *E. coli* colonies under the same conditions would take about 12 hr to appear.(23) If a researcher is extremely pressed for time and needs to rapidly collect data, perhaps using *V. natriegens* for one experiment would save time. Even so, the amount of time thsat *V. natriegens* can save is arguably marginal considering the resources expended for developing novel protocols and introducing a new organism to the laboratory. The inconvenience of replacing *E. coli*, which has been the gold-standard for biologists for decades, with *V. natriegens* is therefore extremely challenging.(15)

E. Coli is a universal model organism and is commercially available in a wide variety of strains, auxotrophs, and mutants. *E. coli* has been intensively studied since 1885 when Theodor Escherich discovered the bacterium.(15) Since the 1940s, non-pathogenic forms of *E. coli* have been used in experiments to resolve key aspects of prokaryotic physiology and behaviour. As time progressed, our understanding of fundamental molecular biological processes grew alongside developments in the *E. coli* system, further reinforcing its use in molecular and cellular biology.(25-27) Categorized, resistant, and harmless, *E. coli* is a manageable bacterium that replicates quickly. With each new protein theorized, gene discovered, or pathway of interest determined, *E. coli* was, and still is, the first organism used to develop an expression system, study metabolism, or generate mutations to illustrate molecular processes. Our comprehension of *E. coli* "omics" (genome, proteome, transcriptome, lipidome, interactome, etc.) is as extensive as the global laboratory use of the organism and has allowed researchers to understand the true effects of any mutation or genetic engineering.(25, 26) Functionally, both *E. coli* and *V. natriegens* are very similar: they are generally the same size, have flagella, and are gram-negative. Yet despite their close relationship, it appears that the widespread availability, functionality, cost effectiveness, and traditions surrounding *E. coli* makes it unlikely that *V. natriegens* will replace *E. coli* in the near-future.

Shortly after the SGI team described *V. natriegens* gene and protein expression systems, the company commercialized VmaxTM Express Competent cells, an electroporation-ready strain of *V. natriegens* equipped with an IPTG-inducible T7 promotor system and a doubling time of 14 min.(23) Vmax now provides scientists with a new choice for molecular cloning and expression studies and can potentially transform bioengineering. As a protein or metabolite expression and production system, *V. natriegens* could greatly improve large-scale yields and operation times. Biopharmaceutical companies use surrogate organisms to produce a number of small-molecule and protein-based drugs in large bioreactors, and *V. natriegens*' faster replication time can substantially increase biomass production, and therefore drug or bioproduct.(26, 27, 28) Although there may not be many cost benefits to using *V. natriegens* at the benchtop level, cost reductions may be realised in large-scale bio-production operations.(28) *V. natriegens* can benefit large-scale bio-refineries looking to cut costs and enhance productivity without compromising quality.

Decreasing the costs and time for bio-product assembly can also benefit the development of personalized medicines.(26, 27) For example, *V. natriegens* is promising in pharmacogenomics and for developing drugs that address genetic based disorders and deficiencies. *V. natriegens*' rapid growth and stability as a genetic vector enhances genomic screening of medical conditions. Through the use of protein expression systems or RNAi/microRNA/CRISPRi, researchers can advance treatments to silence harmful genes, introduce necessary exogenous proteins, and provide faster treatments for patients with novel or uncommon conditions. Perhaps one the most interesting characteristics of *V. natriegens* applicable to the biomedical field is its remarkable ability to secrete proteins directly into the growth medium. As noted by Weinstock and the Synthetic Genomics team, this capability will allow for easier bio-separation processes, thus saving costs downstream.(23)

Conclusion

As for *V. natriegens* itself, much is left to learn about the physiology and mechanisms behind its doubling time. Further understanding its impressive growth phenotype and physiology can uncover genetic and biochemical systems that could increase biofuel crop growth, fight its pathogenic relative *V. cholerae*, or advance understanding of the salt marsh microbiome. For now, a general lack of attention and behavioural description of *V. natriegens* limits the bacterium's adoption in bio-production. Regardless, the biotechnological future looks propitious for *V. natriegens* as scientists have only scratched the surface of this robust microbe.

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