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A rapid in-lab toolbox for the discrimination of *Vibrio natriegens* and *Escherichia coli* cultures

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ABSTRACT

We present a practical toolbox for identifying *Vibrio natriegens* in laboratory cultures, based on observable phenotypes and species-specific PCR. This workflow enables fast discrimination of *V. natriegens* from *Escherichia coli* K-12 and derivatives and microbial contaminants without sequencing or specialized equipment.

Vibrio natriegens is a fast-growing marine bacterium increasingly used in molecular and synthetic biology due to its extremely short doubling time (~10–15 min) and compatibility with common cloning workflows (Hoffart et al., 2017; Lee et al., 2016; Tschirhart et al., 2019; Weinstock et al., 2016). As its adoption rises in academic environments, the risk of cross-contamination with other commonly used microorganisms such as *Escherichia coli* is a practical concern in shared labs or during dual-species experiments. Misidentification of cultures and colonies can compromise plasmid replication, strain integrity, or downstream analysis. While both *E. coli* and *V. natriegens* can support plasmid DNA production, each bacterium requires organism-specific cultivation conditions. Misidentification or unnoticed co-cultivation may lead to inconsistent plasmid yields and process variability, even before downstream processing steps are considered. This poses a challenge for reproducibility and resource efficiency in research and production settings.

In the course of our work to evaluate *V. natriegens* as an *E. coli* alternative for plasmid DNA production, we realized the ease with which such cross-contamination could indeed occur. To address this issue, we developed a rapid and accessible diagnostic toolbox that enables reliable in-lab identification of *V. natriegens* using basic phenotypic traits and targeted PCR to test if species-specific DNA sequences are present, removing the need for sequencing or advanced instrumentation.

The *V. natriegens* Vmax™ X2 strain (Vmax X2 Chemically Competent Cells, Telesis Bio, USA) was transformed with the 3 kbp peGFP plasmid (which drives EGFP expression, as described in supplementary material S1, (Fuchs et al., 2024)). This strain is a derivative of the type strain ATCC 14048 that does not produce the extracellular Dns nuclease. *E. coli* DH5α (Invitrogen™ MAX Efficiency™ DH5α Competent Cells, Thermo Fisher Scientific, USA) was also transformed with the same plasmid. Microbial growth behavior in liquid culture provides an efficient means to discriminate between *V. natriegens* and *E. coli*. When inoculated at an initial 600 nm optical density (OD_{600}) of 0.1 in LB medium (NZYtech, Portugal) supplemented with V2 salts (LB-V2)(0.4 mM NaCl, 4.2 mM KCl, 23.14 mM $MgCl_2$; Sigma-Aldrich, USA), *V. natriegens* Vmax consistently reached an OD_{600} of around 1 (0.97 ± 0.02) within 90 min. In contrast, *E. coli* DH5α only reached an OD_{600} of 0.39 ± 0.02 within 90 min in LB medium (see supplementary material S2, S3). A microbial contaminant that we isolated from *V. natriegens* Vmax and identified by whole-genome sequencing (StabVida, Portugal) as an *E. coli* contaminant strain, reached an OD_{600} of 0.60 ± 0.03 within 90 min of cultivation in LB medium. Furthermore, *V. natriegens* Vmax failed to grow overnight in LB medium lacking V2 salts ($OD_{600} \sim 0$, Fig. 1A). In contrast, *E. coli* strains showed normal growth under the same conditions, indicating that the salt dependency is specific to *V. natriegens*. After centrifugation (13,000 ×*g*, 3 min, 4 °C), cell pellets of *V. natriegens* Vmax displayed a characteristic light pink hue, in contrast to the beige to off-white appearance of the *E. coli* contaminant (Fig. 1C).

On LB-V2 agar, *V. natriegens* Vmax colonies appeared small to medium, round, and slightly glossy, with a tendency to merge with each

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other. *E. coli* DH5α produced small, flat, glassy colonies, whereas *E. coli* contaminant formed larger, irregular colonies with a smooth surface (Fig. 1A, see supplementary material S7). *V. natriegens* and *E. coli* stained Gram-negative and showed typical short rod-shaped morphology under brightfield microscopy (see supplementary material S4).

Since *V. natriegens* carries a gene encoding for cytochrome *c* oxidase subunit 1 (*ccoN*), which is absent in *E. coli*, a rapid colorimetric oxidase test (Sigma-Aldrich, USA) was used to discriminate the two bacteria by applying test strips directly to overnight-grown colonies. *V. natriegens* Vmax produced an oxidase-positive result, turning the strip blue within 30 s, whereas both *E. coli* DH5α and the *E. coli* contaminant were oxidase-negative (Fig. 1A, (Fuchs et al., 2024)). This phenotypic test reflects the presence of cytochrome *c* oxidase activity, which in *V. natriegens* is typically associated with the *ccoNOP* gene cluster (Cosseau and Batut, 2004). This oxidase is part of the respiratory chain of many bacteria, being responsible for electron transfer to the final acceptor, catalyzing the reduction of oxygen to water. The dye in the strips can be reduced by the oxidase, resulting in the change of color (Madigan et al., 2018). In contrast, *E. coli* strains such as DH5α do not possess this specific oxidase, resorting to a different set of terminal oxidases and reductases and, as such, give a negative test outcome (Unden and Dünnwald, 2008).

To further differentiate *V. natriegens* from *E. coli* K-12 and its derivative DH5α, we established a dual-marker PCR targeting the *ccoN* gene (cytochrome *c* oxidase subunit I), which is specific for *V. natriegens*, and the truncated *lacZ* gene, which is present in *E. coli* K-12 and DH5α, but absent in *V. natriegens* Vmax. The choice of *ccoN* as a molecular target reflects its functional role in the cytochrome *c* oxidase complex, directly corresponding to the oxidase-positive phenotype of *V. natriegens*. Primer sequences used to target *ccoN* and *lacZ* alongside the expected amplicon size are presented in Table 1.

PCR reactions were prepared using Supreme NZYTAQ II 2× Colourless Master Mix (NZYtech, Portugal), with 0.2 µM of each primer, 5 µL of crude lysate from an $OD_{600} = 2.0$ culture (centrifuged and resuspended in 500 µL PCR-grade water), and exposed to a PCR program consisting of 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 57.5 °C for 30 s, 72 °C for 30 s; followed by 1 min at 72 °C. The annealing temperature of 57.5 °C, which was chosen based on primer melting temperatures and available recommendations, was found to produce clear, specific bands without further optimization. Amplicons were analyzed on a 1 % agarose gel. *V. natriegens* Vmax samples produced the *ccoN* band exclusively, while *E. coli* K-12 and its derivative DH5 exhibited the *lacZ* amplicon. The *E. coli* contaminant strain exhibited no observable amplification in both cases (see supplementary material S6). While the

Table 1
Primer sequences and expected amplicon sizes for PCR targeting of the *ccoN* gene in *V. natriegens* and the *lacZ* gene in *E. coli* K-12 and DH5α.

Gene	Primers	Amplicon
ccoN (*V. natriegens*)	TTGGTATGGCTGTTGGTG AACCGTACCGATTGTTGC	1082 bp
lacZ (*E. coli K-12 and DH5α*)	CCGATATTATTTGCCCGATG ACTTCAACATCAACGGTAATC	1129 bp

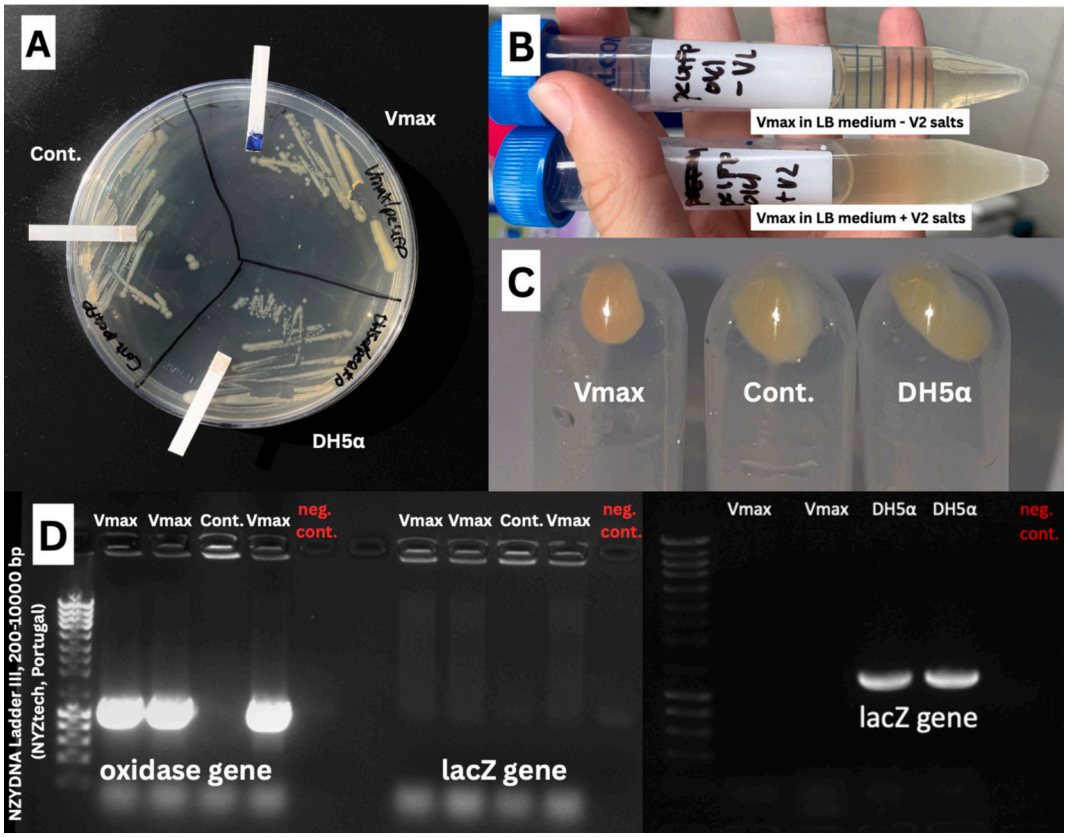

Fig. 1. Phenotypic and molecular characteristics used for the identification of *Vibrio natriegens* cultures. (A) Cytochrome c oxidase test on LB-V2 agar plates. Only *V. natriegens* Vmax yields a positive result (blue strip), while both *E. coli* contaminant (Cont.) and *E. coli* DH5α are oxidase negative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) (B) Growth in LB-V2 and LB medium. *V. natriegens* Vmax grows only in the presence of V2 salts. (C) Pellet color after overnight LB (LB-V2 for *V. natriegens*) culture: pink hue for *V. natriegens*, beige hue for *E. coli* contaminant (Cont.) and *E. coli* DH5α. (D) Agarose gel analysis of species-specific PCR products. *ccoN* (oxidase gene) is amplified only in *V. natriegens*. *lacZ* (lacZ gene) is detected only in *E. coli* DH5α. No amplification is observed in the *E. coli* contaminant (Cont.) due to missing primer-binding site. NZYDNA ladder III, 200–10,000 bp (NZYtech, Portugal); neg. Cont.: negative control corresponds to a no-template control (NTC).

lack of amplification for the *ccoN* gene was expected, we expected amplification of the *lacZ* gene for both *E. coli* DH5α and the *E. coli* contaminant. A more detailed analysis of the sequencing files showed that the *lacZ* gene of the contaminant was truncated when compared to the gene in *E. coli* K-12 substr. MG1655 (NCBI Reference Sequence: NZ_CP010444.1). A significant portion of its N-terminal was missing including the F primer annealing site (see supplementary material S5). This observation underscores the value of combining phenotypic and molecular markers for robust strain discrimination in routine settings (Fig. 1D).

In summary, the compact diagnostic workflow presented here describes the rapid identification and differentiation of *V. natriegens* Vmax from *E. coli* laboratory strains (K-12 and derivatives, e.g. DH5α). Requiring only minimal equipment (e.g. a thermocycler), this simple yet effective quality control method is well-suited for laboratories working with both species, offering a practical means to monitor and mitigate cross-contamination.

CRediT authorship contribution statement

Lara S. Möller: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **José M.M. Santos:** Writing – review & editing, Methodology, Investigation, Formal analysis. **A. Rita Silva-Santos:** Writing – review & editing, Supervision, Resources, Methodology, Investigation. **Duarte M.F. Prazeres:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2025.107273.

Data availability

No data was used for the research described in the article.

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