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RESEARCH ARTICLE

Investigation of exopolysaccharide formation and its impact on anaerobic succinate production with *Vibrio natriegens*

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Abstract

Vibrio natriegens is an emerging host for biotechnology due to its high growth and substrate consumption rates. In industrial processes typically fed-batch processes are applied to obtain high space-time yields. In this study, we established an aerobic glucose-limited fed-batch fermentation with the wild type (wt) of *V. natriegens* which yielded biomass concentrations of up to 28.4 $g_X L^{-1}$. However, we observed that the viscosity of the culture broth increased by a factor of 800 at the end of the cultivation due to the formation of 157 ± 20 mg exopolysaccharides (EPS) L^{-1}. Analysis of the genomic repertoire revealed several genes and gene clusters associated with EPS formation. Deletion of the transcriptional regulator *cpsR* in *V. natriegens* wt did not reduce EPS formation, however, it resulted in a constantly low viscosity of the culture broth and altered the carbohydrate content of the EPS. A mutant lacking the *cps* cluster secreted two-fold less EPS compared to the wt accompanied by an overall low viscosity and a changed EPS composition. When we cultivated the succinate producer *V. natriegens* Δ*lldh* Δ*dldh* Δ*pfl* Δ*ald* Δ*dns*::pyc_{Cg} (Succ1) under anaerobic conditions on glucose, we also observed an increased viscosity at the end of the cultivation. Deletion of *cpsR* and the *cps* cluster in *V. natriegens* Succ1 reduced the viscosity five- to six-fold which remained at the same level observed at the start of the cultivation. *V. natriegens* Succ1 Δ*cpsR* and *V. natriegens* Succ1 Δ*cps* achieved final succinate concentrations of 51 and 46 g L^{-1} with a volumetric productivity of 8.5 and 7.7 g_{Suc} L^{-1} h^{-1}, respectively. Both strains showed a product yield of about 1.4 mol_{Suc} mol_{Glc}^{-1}, which is 27% higher compared with that of *V. natriegens* Succ1 and corresponds to 81% of the theoretical maximum.

INTRODUCTION

Exopolysaccharides (EPS) are high-molecular weight carbohydrate polymers that are secreted into the extracellular environment without any attachment to the cell or as capsular polysaccharides which are associated with the cell surface (Cescutti, 2010). They are produced by various microorganisms like bacteria, yeasts and fungi (Barcelos et al., 2020). EPS are essential for biofilm formation and play an important role in pathogenicity. They protect the cell from environmental stress by altering the physical and chemical micro-environment

around the cell and are essential for the adhesion to surfaces and other organisms as well as for the production of aggregates in a marine environment (reviewed in Moradali & Rehm, 2020; Nichols et al., 2005). EPS are grouped into homopolysaccharides, which consist of only one monosaccharide, and heteropolysaccharides. The latter consists of various monosaccharides and represents the majority of EPS secreted by microbes (Barcelos et al., 2020). The structure of heteropolysaccharides is often composed of hexoses, pentoses, amino sugars and uronic acids and the EPS may also contain non-sugar organic and inorganic substituents such as succinic and acetic acid, sulfates and phosphates (Kenne & Lindberg, 1983). EPS have a wide range of applications in the medical, pharmaceutical and food industries, as well as in wastewater treatment (Barcelos et al., 2020). Consequently, EPS biosynthesis and its regulation is studied in detail to eventually engineer production hosts towards tailor-made EPS with improved or new physiochemical properties (Schilling et al., 2020). However, EPS often represent an unwanted byproduct in microbial production processes. EPS formation may change the bioreactor broth from a Newtonian to a non-Newtonian fluid accompanied with an increased viscosity. This negatively affects the mixing of the broth and leads to a heterogeneous system with a reduced mass transfer which in turn hinders a sufficient heat transfer and oxygen supply. Moreover, EPS formation may negatively affect downstream processing (reviewed in Seviour et al., 2011).

Vibriae is model organism for studying EPS synthesis, biofilm formation and quorum sensing. Quorum sensing, the cell-to-cell communication, occurs in many *Vibrios* such as *Vibrio harveyi* (Henke & Bassler, 2004), *Vibrio cholera* (Jung et al., 2016), *Vibrio parahaemolyticus* (Kalburge et al., 2017) and *Vibrio fischeri* (Ball et al., 2017) and is often closely linked to biofilm formation and EPS production (Yildiz & Visick, 2009). In *V. parahaemolyticus*, the *cps* gene cluster is required for the production of capsular polysaccharides (Güvener & McCarter, 2003). The *syp* genes (symbiosis polysaccharide genes) in *V. fischeri* are related to biofilm formation and play an important role in the symbiotic colonization of the Hawaiian bobtail squid *Euprymna scolopes*. Homologous clusters are also present in *V. parahaemolyticus*, *V. vulnificus* and *V. diabolicus* (Yip et al., 2005). In the latter, the *syp* cluster can be assigned to the synthesis of the valuable EPS HE800 which is made from equal parts of uronic acid and hexosamine and is known for its bone regeneration capability without inducing inflammatory reactions (Goudenège et al., 2014; Zanchetta et al., 2003). Lebellenger et al. (2018) identified in 72 out of 103 genome sequences of Vibrionaceae genetic elements similar to the *syp* cluster of *V. diabolicus*.

One such strain harbouring a *syp* cluster is *Vibrio natriegens*, which is considered as a promising host for biotechnology due to its outstandingly high growth and biomass specific substrate uptake rates (Hoffart et al., 2017). *V. natriegens* is a facultatively anaerobic marine bacterium which possesses a versatile metabolism and can utilize a variety of substrates as carbon and energy sources. This Gram-negative non-pathogenic γ-proteobacterium is easy to handle in the lab and a genetic engineering toolbox for *V. natriegens* is readily available (reviewed in Hoff et al., 2020; Stukenberg et al., 2021; Teufel et al., 2022; Thoma & Blombach, 2021). This toolbox has been applied to delete two prophage regions (VNP1 and VNP2), which are induced under stress but also under standard conditions, to engineer a genetically more stable platform strain for future metabolic engineering studies (Pfeifer et al., 2019). Moreover, *V. natriegens* has been utilized as host for molecular biology (Lee et al., 2016; Weinstock et al., 2016), (cell-free), protein synthesis (Failmezger et al., 2018; Schleicher et al., 2018; Sun et al., 2023) and for the production of, e.g., 2,3-butanediol (Erian et al., 2020; Meng et al., 2022), succinate (Thoma et al., 2021), 1,3-propanediol (Zhang et al., 2021) and alanine (Hoffart et al., 2017).

The ability of *V. natriegens* to form biofilms on metallic surfaces with EPS as an adhesive has been described (Cheng et al., 2010; Dong et al., 2016). However, further investigations on EPS formation with *V. natriegens* are not available so far. In this study, we show that *V. natriegens* forms EPS in aerobic glucose-limited fed-batch fermentations as well as under anaerobic conditions with resting succinate producing cells. We analysed mutants lacking genes and gene clusters putatively related to EPS synthesis and studied the effect of the deletions on EPS formation and composition as well as on the rheology of the culture broth and its impact on anaerobic succinate production.

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

All bacterial strains and plasmids used in this work are listed in Table 1. For permanent storage, all strains were preserved at −80°C as 30% (v v^{-1}) glycerol stocks. For preparation of the glycerol stocks, cells were cultivated overnight (O/N) in 5 mL of 2× YTN medium (Sambrook & Russell, 2001). Thereafter, an appropriate amount of the cell suspension was mixed with glycerol and immediately frozen at −80°C.

Cultivation media and conditions

Escherichia coli was grown routinely in 2× YT containing 16 g tryptone L^{-1}, 10 g yeast extract L^{-1} and 10 g NaCl L^{-1} (Sambrook & Russell, 2001). For cultivation of

TABLE 1 Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli DH5α	F⁻ ϕ80*lacZ*ΔM15 Δ(*lacZYA-arg*F)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA96 relA1 phoA*	Hanahan (1983)
E. coli S17-1 λ*pir*	F⁻ *thi pro hsdR hsdM*⁺ *recA* RP4-2-Tc::Mu-Km::Tn7 λ*pir* Tp^R Sm^R	Simon et al. (1983)
E. coli Π3813	F⁻ *lacI*^{q} *thi-1 supE44 endA1 recA1 hsdR17 gyrA462 zei-298*::Tn*10* Δ*thyA*::(*erm-pir116*) Tc^R Em^R	Le Roux et al. (2007)
E. coli β3914	F⁻ RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*) *gyrA462 zei-298*::Tn*10* Km^R Em^R Tc^R	Le Roux et al. (2007)
V. natriegens wt	Wild-type strain DSM 759 (ATCC 14048)	German Collection of Microorganisms and Cell Cultures
V. natriegens Δ*cpsR*	*V. natriegens* wt with deletion of the *cpsR* gene (PN96_10960)	This work
V. natriegens Δ*wbfF*	*V. natriegens* wt with deletion of the *wbfF* gene (PN96_12195)	This work
V. natriegens Δ*cps*	*V. natriegens* wt with deletion of the genomic region PN96_14980 to PN96_15050	This work
V. natriegens Δ*syp* Δ*cps*	*V. natriegens* Δ*cps* with additional deletion of the genomic region PN96_04590 to PN96_04675	This work
V. natriegens Succ1	*V. natriegens* Δ*lldh* Δ*dldh* Δ*pfl* Δ*ald* Δ*dns*::pyc_{Cg}	Thoma et al. (2021)
V. natriegens Succ1 Δ*cpsR*	*V. natriegens* Succ1 with deletion of the *cpsR* gene (PN96_10960)	This work
V. natriegens Succ1 Δ*cps*	*V. natriegens* Succ1 with deletion of the genomic region PN96_14980 to PN96_15050	This work
Plasmids		
pDM4	$oriV_{R6K}$, $oriT_{RP4}$, *sacB*, Cm^R	Milton et al. (1996)
pDM4-Δ*cpsR*	pDM4 plasmid carrying 500 bp homologous sequences upstream and downstream of the *cpsR* gene	This work
pSW7848	$oriV_{R6K}$, $oriT_{RP4}$, *araC*-P_{BAD}-*ccdB*, Cm^R	Val et al. (2012)
pSW7848-Δ*wbfF*	pSW7848 plasmid carrying 500 bp homologous sequences upstream and downstream of the *wbfF* gene	This work
pST_116	*oriColE1*, *tfoX*, *cas9*, *acrIIA4*, *sfgfp*, gRNA scaffold, *tetR*, *lacI*, Cm^R	Stukenberg et al. (2021)
pST_116-Δ*syp*	pST_116 with *sfgfp* exchanged for gRNA spacer targeting the *syp* region	This work
pST_116-Δ*cps*	pST_116 with *sfgfp* exchanged for gRNA spacer targeting the *cps* region	This work

V. natriegens, the NaCl concentration was increased to 15 g L^{-1} (2 × YTN). For cloning, *V. natriegens* was grown in LBv2 containing 10 g tryptone L^{-1}, 5 g yeast extract L^{-1}, 15 g NaCl L^{-1}, 5 g $MgCl_2 \times 6\ H_2O$ L^{-1}, 0.3 g KCl L^{-1} (adapted from Weinstock et al., 2016). Plates contained additionally 15 g agar L^{-1}.

For cultivation, cells from a glycerol stock were streaked out on a complex medium agar plate which was incubated O/N at 37°C. Thereafter, a single colony was used to inoculate 5 mL of 2× YT (*E. coli*) or 2× YTN supplemented with 50 mM $MgCl_2$ (*V. natriegens*) in a glass tube which was incubated for 8–10 h O/N at 37°C on an orbital shaker (Ø 25 mm, Multitron®2; INFORS GmbH) at 180 rpm. On the following day, 500 μL of the *V. natriegens* culture was used to inoculate a 500 mL baffled shaking flask with 50 mL of VN minimal medium consisting of the following compounds: 5 g $(NH_4)_2SO_4$ L^{-1}, 15 g NaCl L^{-1}, 1 g K_2HPO_4 L^{-1}, 1 g KH_2PO_4 L^{-1}, 21 g 3-(*N*-morpholino) propanesulfonic acid (MOPS) L^{-1}, 0.01 g $CaCl_2 \times H_2O$ L^{-1}, 0.25 g $MgSO_4 \times 7H_2O$ L^{-1}, 16.4 mg $FeSO_4 \times 7H_2O$ L^{-1}, 10 mg $MnSO_4 \times H_2O$ L^{-1}, 0.31 mg $CuSO_4 \times 5H_2O$ L^{-1}, 1 mg $ZnSO_4 \times 7H_2O$ L^{-1}, and 0.02 mg $NiCl_2 \times 6H_2O$ L^{-1} (Hoffart et al., 2017). The pH was adjusted to 7.5 using 5 M KOH. The medium was additionally supplemented with 10 g glucose L^{-1} and the cells were grown aerobically for 2.5 h at 37°C on the orbital shaker at 180 rpm. Afterwards, the cells were harvested and used to inoculate a bioreactor culture. Fermentations were performed with a DASGIP Parallel Bioreactor System (Eppendorf) with a vessel volume of 2 L.

If necessary, chloramphenicol was added to final concentrations of 15 μg mL^{-1} for *E. coli* and 3 and 6 μg mL^{-1} for *V. natriegens* in liquid and solid medium, respectively.

Glucose-limited fed-batch fermentations

For glucose-limited fed-batch fermentations, a shaking flask preculture in VN medium was harvested

by centrifugation (10 min, room temperature (RT), 4347 × g). The cells were resuspended in 0.9% NaCl (w v^{-1}) and an appropriate volume was used to inoculate the bioreactor to an optical density of 0.1 measured at 600 nm (OD_{600}). Cultivation was performed with 1 L VN minimal medium (without MOPS). The concentration of $MgSO_4 \times 7H_2O$ was raised to 1.2 g L^{-1}. Additionally, the medium was supplemented with 0.1 g Fe(III)citrate × H_2O L^{-1} and 6 g glucose L^{-1}. An exponentially increasing feed was started shortly before glucose depletion containing 550 g glucose L^{-1}, 1.2 g $MgCl_2$ L^{-1}, and 15 g NaCl L^{-1} to achieve a growth rate of 0.1 h^{-1}. The temperature was set to 37°C. Pressurized air at 1 vvm was used to aerate the bioreactor. The agitation rate was adjusted between 400 and 1500 rpm during the process to control dissolved oxygen (DO) above 50%. A polarographic probe (Mettler Toledo) was used to determine the DO concentration in the bioreactor broth. The pH was maintained at 7 using a two-sided pH regulation using 13.3 M NH_4OH and 1.1 M H_3PO_4. The value was monitored by a standard pH probe (405-DPAS-SC-K8S/325; Mettler Toledo).

Zero-growth anaerobic bioprocess for succinate production

For anaerobic succinate production, cells from nine shaking flask precultures in 2× YTN medium were used to inoculate three bioreactors containing 1.5 L 2 × YTN medium supplemented with 50 mM $MgCl_2$, respectively. During the cultivation, the pH was controlled at 7.5 and the temperature was adjusted to 37°C. To obtain microaerobic conditions, the aeration rate was set to 0.5 vvm and a constant agitation of 600 rpm was applied. After 2.5 h, the biomass of all bioreactors was harvested by centrifugation (10 min, RT, 4347 × g). The cell pellet was resuspended in 400 mL VN minimal medium (without MOPS) initially containing 100 g glucose L^{-1} and 150 mM $KHCO_3$ and used to inoculate the main bioreactor culture at a cell dry weight (CDW) of approximately 17 $g_X L^{-1}$. To achieve anaerobic conditions, no aeration was applied and the stirrer speed was kept constant at 180 rpm. The pH was regulated to 7.5 and the temperature to 37°C. All parameters were monitored as described above. After inoculation, a linear $KHCO_3$ feed was applied at 100 mM h^{-1}.

Construction of strains

Gene identification was performed using BLASTN and BLASTP (Altschul et al., 1997). Genome reference and gene identifiers are used from GenBank assembly accession GCA_001456255.1 (Lee et al., 2016). Oligonucleotides used in this study were ordered from Sigma-Aldrich Chemie GmbH and are listed in Table S1. All the enzymes were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany) and used according to the manufacturer's recommendation. NucleoSpin Microbial DNA and NucleoSpin Plasmid kit (Macherey-Nagel) were used for chromosomal and plasmid DNA isolation. PCR fragment purification was performed with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). The kits were used according to the manufacturer's protocol. Competent *E. coli* cells for electroporation were prepared as described by Dower et al. (1988) using 2× YT medium. Competent *V. natriegens* cells for electroporation were prepared in LBv2 medium as described by Weinstock et al. (2016).

Deletions of *cpsR* and *wbfF* were performed with suicide plasmid derivatives of pDM4 (Milton et al., 1996) and pSW7848 (Val et al., 2012), respectively. DNA fragments of 500 bp upstream and downstream of the target gene were amplified by PCR using the primer pairs cpsR1/cpsR2, cpsR3/cpsR4, wbfF1/wbfF2 and wbfF3/wbfF4 and inserted into the respective XbaI- and SalI-linearized plasmid by Gibson assembly (Gibson, 2011). *E. coli* DH5α was used as cloning host for work with pDM4 derivatives and *E. coli* π3813 for pSW7848 derivatives. Resulting plasmids were isolated and verified by sequencing (Microsynth Seqlab GmbH). Conjugation into *V. natriegens* was performed as described before by Hoffart et al. (2017) with LBv2 instead of LBN and BHIN medium. *E. coli* S17-1 λpir and *E. coli* β3914 were used for conjugal transfer of pDM4-Δ*cpsR* and pSW7848-Δ*wbfF*, respectively. Deletions by homologous recombination were generated by incubating transformed cells for 6 h in LBv2 at 30°C. Then, the cell suspension of a dilution series up to 10^{-5} cells was subsequently plated on LBv2 with 10% (w v^{-1}) sucrose for counter selection of pDM4 derivatives (Milton et al., 1996) or with 0.2% (w v^{-1}) arabinose for counter selection of pSW7848 derivatives (Val et al., 2012). Plates were incubated 24 h at RT and single colonies checked for successful plasmid disintegration and target deletion.

Deletions of the *syp* and *cps* regions were performed with the NT–CRISPR method described by Stukenberg et al. (2021). 3000 bp flanks were amplified upstream and downstream of the target regions with the primer pairs syp-1/syp-2, syp-3/syp-4, cps-1/cps-2 and cps-3/cps-4. The flanks of each target were fused by overlap extension PCR to create the tDNAs. gRNAs were designed in the software Benchling following the scores based on Doench et al. (2014) and Hsu et al. (2013). Transformation and counter selection were performed as described with the deviation of using 0.5 mM IPTG for *tfoX* induction, 200 ng tDNA and plating was done with 10^0 and 10^{-1} dilutions. Deletions were verified by colony PCR using Hot Start Taq polymerase and syp-col1/syp-col2/syp-col3 or cps-col1/cps-col2/cps-col3 primer combinations, respectively. Template DNA was provided by resuspending cell material in 10 µL H_2O

and subsequential incubation at 95°C for 15 min. 1 μL of the suspension was used in a 10 μL colony PCR volume. Plasmid curing was performed as described by Stukenberg et al. (2021).

Analytics

Quantification of sugars and acids

For metabolite analysis, samples of the bioreactor broth were prepared by centrifugation (10 min, RT, 4347 × g). The supernatant was measured with a high-performance liquid chromatography (HPLC) to quantify organic sugars and acids such as glucose, lactate, succinate, pyruvate and acetate. For this purpose, an Agilent 1260 infinity II series device (Agilent Technologies) equipped with a Hi-Plex H column (7.7 × 300 mm, 8 μm) and protected by a Hi-Plex H guard cartridge (3.0 × 5.0 mm, 8 μm) was used. Analytes were separated isocratically with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL min^{-1} at 50 °C and detected by a refractive index detector (RID). Metabolite concentrations were calculated by comparing the peak area of the sample with the peak area of a 7-point external standard ranging from 1 to 200 mM (Siebert et al., 2021).

EPS were quantified directly from the bioreactor broth by the high throughput 1-phenyl-3-methyl-5-pyrazolone (HT–PMP) method coupled with an Ultra High-performance liquid chromatography with ultra violet and electrospray ionization ion trap tandem mass spectrometry (UHPLC–UV–ESI–MS/MS) as described by Rühmann et al. (2014, 2015). For sample preparation, the bioreactor broth was centrifuged (15,871 × g, 10 min) and the centrifugation steps were repeated until a clear supernatant was obtained. Subsequently, 30 μL of supernatant were placed in the center of each well of a gel-filtration plate (96-well SpinColumn G-25, Harvard Apparatus) that had been equilibrated by washing three times with 150 μL water. The plate was centrifuged at 1000 × g for 2 min at 20°C. Hydrolysis was performed by incubating 20 μL EPS solution with 20 μL 4 M TFA at 121°C for 90 min in a 96-well PCR plate (781350; Brand). After cooling to RT, hydrolysis was stopped by adding 3.2% NH_4OH solution, increasing the pH to slightly alkaline conditions. Sugar standards were prepared with the neutralized TFA-hydrolysis matrix. Derivatization of samples/standards was performed by mixing the 25 μL hydrolysate with the 75 μL derivatization reagent, containing a mix of 0.1 M PMP in MeOH and 0.4% NH_4OH solution in ratio 2:1 (v v^{-1}). After incubation for 100 min at 70°C in a PCR-cycler (Biorad Mycycler), 20 μL derivatized sample was mixed with 130 μL 19.23 mM acetic acid. Samples were filtered (0.2 μm Supor; Pall Corporation) into a microtiter plate, which was sealed with a silicon cap mat (Whatman 7704-0105). Analysis was performed using UHPLC equipped with a reverse phase column (Gravity C18; Macherey-Nagel) tempered to 50°C and an UV detector (245 nm). Mobile phase A contained a mixture of 5 mM ammonium acetate buffer (pH 5.6) with 15% (w w^{-1}) acetonitrile and mobile phase B contained pure acetonitrile following the programmed gradient at a flow rate of 0.6 mL min^{-1}. Before accessing ESI-MS, the flow was split 1:20 (Accurate-Post-Column-Splitter; Dionex).

The limit of detection was usually between 0.2 and 0.6 mg L^{-1} and the limit of quantification varied around 0.5–2 mg L^{-1}. For a more detailed description see Rühmann et al. (2015).

Determination of growth parameters

OD_{600} measurements of the cultures were performed using a spectrophotometer (Ultrospec 10 cell density meter; Harvard Biochrom). The cell dry weight (CDW) in g_X L^{-1} was calculated by the conversion factor 0.3 × OD_{600}. The growth rate μ was obtained by the slope of a linear regression curve of the semi logarithmic plot of CDW versus process time during the exponential growth phase. The biomass yield $Y_{X/S}$ was determined by linear regression of the CDW plotted against the respective glucose concentration for exponentially growing cells. The biomass-specific glucose consumption rate q_S was calculated by using the equation $q_S = \mu / Y_{X/S}$.

The oxygen transfer rate (OTR) was calculated by a gas mass balance of the exhaust gas. For this purpose, a gas analyser GA4 (Eppendorf) with a galvanic measurement cell was directly connected to the bioreactors and used to detect oxygen and carbon dioxide.

Rheological measurements

For rheological measurements, the reactor broth was centrifuged (10 min, RT, 15,871 × g) and the supernatant was stored at −20°C until measurement. The viscosity was determined at 20°C using a MCR302 (Anton-Paar) rotational rheometer equipped with a TEK150 chamber and a CP50-1 measuring cell.

RESULTS

Establishment of a glucose-limited fed-batch process

To obtain high space time yields in industrial processes, typically fed-batch processes are applied. Here, we established an aerobic glucose-limited fed-batch

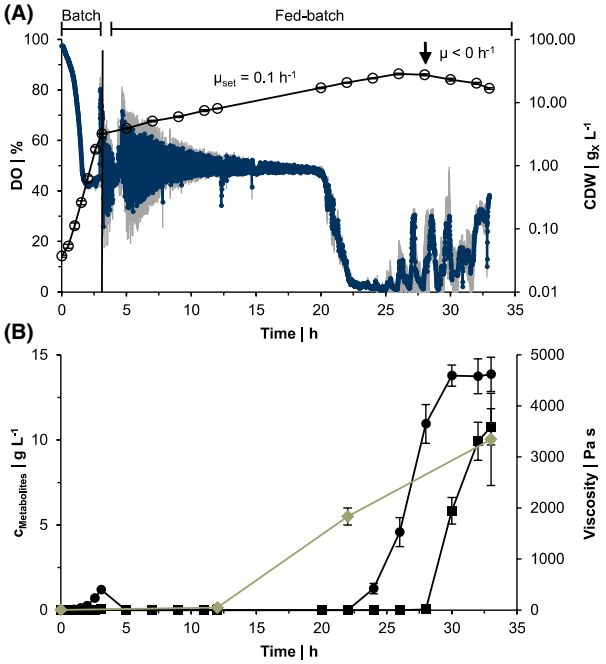

FIGURE 1 Glucose-limited fed-batch process with *Vibrio natriegens* wt. The courses of cell dry weight (hollow circle) and dissolved oxygen (dark blue line) are shown in (A). The concentrations of acetate (circles), pyruvate (squares), and viscosity (green line) measured at a shear rate of 0.1 s^{-1} are depicted in (B). Results shown are means and standard deviations (error bars for cell dry weight and metabolites; grey line for dissolved oxygen) of three independent experiments. The arrow indicates a deviation from the growth rate μ_{set}.

fermentation with *V. natriegens* wt comprising an unlimited batch phase and a feed phase (Figure 1; Table 2). During the batch phase, *V. natriegens* grew at maximum growth rate of 1.67 ± 0.01 h^{-1} and showed a biomass yield ($Y_{X/S}$) of 0.49 ± 0.01 $g_X g_S^{-1}$ and a biomass specific glucose consumption rate (q_S) of 3.44 ± 0.10 $g_S g_X^{-1} h^{-1}$ (Table 2). Within the batch phase up to 1.2 g acetate L^{-1} accumulated, which was subsequently reutilized in second phase of the process (Figure 1B). After around 3 h, glucose was almost depleted and the fed-batch phase was initiated by adding an exponentially increasing glucose feed. The feeding rate was adjusted for glucose-limited growth to obtain a growth rate μ_{set} of 0.1 h^{-1}. During the fed-batch phase, a constant μ of 0.10 ± 0.01 h^{-1} was maintained for 20 h. After about 24 h the DO dropped below 5% and *V. natriegens* started to secrete acetate into the medium. Subsequently, μ decreased and a decline in CDW was observed after 28 h. At this time point, pyruvate began to accumulate as well. Maximum concentrations of 13.78 ± 0.6 g acetate L^{-1}, 10.77 ± 1.1 g pyruvate L^{-1} and 28.40 ± 0.75 g_X biomass L^{-1} were reached (Figure 1A,B; Table 2). Rheological measurements revealed an increasing viscosity (η) of the reactor broth over the course of the fermentation. A maximum viscosity η_{max} of 3344 ± 903 mPa s (at a shear rate $\dot{\gamma}$ of 0.1 s^{-1}) was measured after 33 h which corresponds to an 800-fold increase compared to η at the start of the process (Figure 1B; Table 2).

TABLE 2 Growth parameters, substrate and metabolite concentrations and the viscosity for fed-batch fermentations of *Vibrio natriegens* wt and the given deletion mutants.

	V. natriegens				
Phase KPI	**wt**	**Δ*cpsR***	**Δ*wbfF***	**Δ*cps***	**Δ*syp* Δ*cps***
Batch phase					
$\mu \mid h^{-1}$	1.67 ± 0.01	1.63 ± 0.03	1.57 ± 0.09	1.66 ± 0.05	1.52 ± 0.08
$Y_{X/S} \mid g_X g_S^{-1}$	0.49 ± 0.01	0.45 ± 0.01	0.36 ± 0.01	0.46 ± 0.05	0.42 ± 0.03
$q_S \mid g_S g_X^{-1} h^{-1}$	3.44 ± 0.10	3.66 ± 0.05	4.41 ± 0.33	3.66 ± 0.27	3.61 ± 0.09
Fed-batch phase					
$\mu \mid h^{-1}$	0.10 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
$Y_{X/S} \mid g_X g_S^{-1}$	0.36 ± 0.01	0.37 ± 0.03	0.40 ± 0.01	0.32 ± 0.02	0.36 ± 0.03
$q_S \mid g_S g_X^{-1} h^{-1}$	0.27 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.31 ± 0.02	0.28 ± 0.01
$CDW_{max} \mid g_X L^{-1}$	28.4 ± 0.8	27.6 ± 1.7	26.6 ± 2.0	24.8 ± 0.8	24.2 ± 0.8
$\eta_{max} \mid$ mPa s	3344 ± 903	3 ± 1	1054 ± 203	5 ± 1	7 ± 1
$OTR_{max} \mid$ mmol $L^{-1} h^{-1}$	132 ± 3	165 ± 2	169 ± 4	161 ± 13	173 ± 6
$c_{Acetate,max}$ g L^{-1}	13.78 ± 0.6	6.97 ± 0.2	5.18 ± 1.6	7.54 ± 0.9	5.41 ± 0.5
$c_{Pyruvate,max}$ g L^{-1}	10.77 ± 1.1	0.38 ± 0.5	2.67 ± 0.5	0.87 ± 1.2	0.06 ± 0.1
$c_{EPS,max}$ mg L^{-1}	157 ± 20	176 ± 8	299 ± 69	82 ± 14	83 ± 7

Note: Results given are means and standard deviations of three independent biological replicates.

Genomic repertoire for EPS formation

We speculated that the observed changes in rheology were caused by the formation of exopolysaccharides (EPS). Therefore, we analysed the genome and identified several genes and gene clusters potentially involved in EPS formation (Figure 2). CpsR of *V. parahaemolyticus* and the homologue VpsR of *V. cholera* are described as positive transcriptional regulators of EPS formation (Yildiz et al., 2001). The nucleotide sequence of *vpsR* of *V. cholerae* (VC_0665; Heidelberg et al., 2000) and *cpsR* of *V. parahaemolyticus* (AY216912; Güvener & McCarter, 2003) showed a 71% and 81% identity, respectively, to PN96_10960 of *V. natriegens*, henceforth called *cpsR*. Similarly, the nucleotide sequence of *cpsA* as part of the *cps* cluster responsible for EPS formation in *V. parahaemolyticus* (Jensen et al., 2013) had 79% identity with PN96_14980 and revealed a genomic region (CPS) resembling the *cps* cluster in *V. natriegens* (Figure 2). BLASTP analysis of SypK of *V. fischeri* (VF_A1030; Ruby et al., 2005) resulted in a hit of 35% identity with PN96_0426 and the genomic region showed a *syp*-like cluster in *V. natriegens*. Furthermore, the genome of *V. natriegens* harbours the *wbfF* gene which has already been described to be involved in the biosynthesis of capsular polysaccharides (Dalia et al., 2017). Surrounding this gene, several genes annotated to be part of polysaccharide and lipopolysaccharide synthesis can be found, including a number of sugar transferases and slightly upstream a cluster for the general secretion pathway, a type II secretion system for polysaccharides (Ali et al., 2000; Johnson et al., 2014). The *syp* and *cps* cluster as well as *wbfF* and *cpsR* were selected as potential targets to impact EPS formation in *V. natriegens* in this study.

Characterization of strains with deletion of genes associated with EPS formation

To investigate which genes or gene clusters are involved in EPS formation under the applied conditions, we deleted the *cpsR* and *wbfF* genes from start to stop codon as well as the *cps* and *syp* clusters from the start codon of the first gene to the stop codon of the last gene in *V. natriegens* wt. With the resulting strains *V. natriegens* Δ*cpsR*, *V. natriegens* Δ*wbfF*, *V. natriegens* Δ*cps*, and *V. natriegens* Δ*syp* Δ*cps* we performed glucose-limited fed-batch fermentations as described above and analysed the growth parameters, substrate consumption, metabolite formation and the viscosity (Figure 3; Table 2).

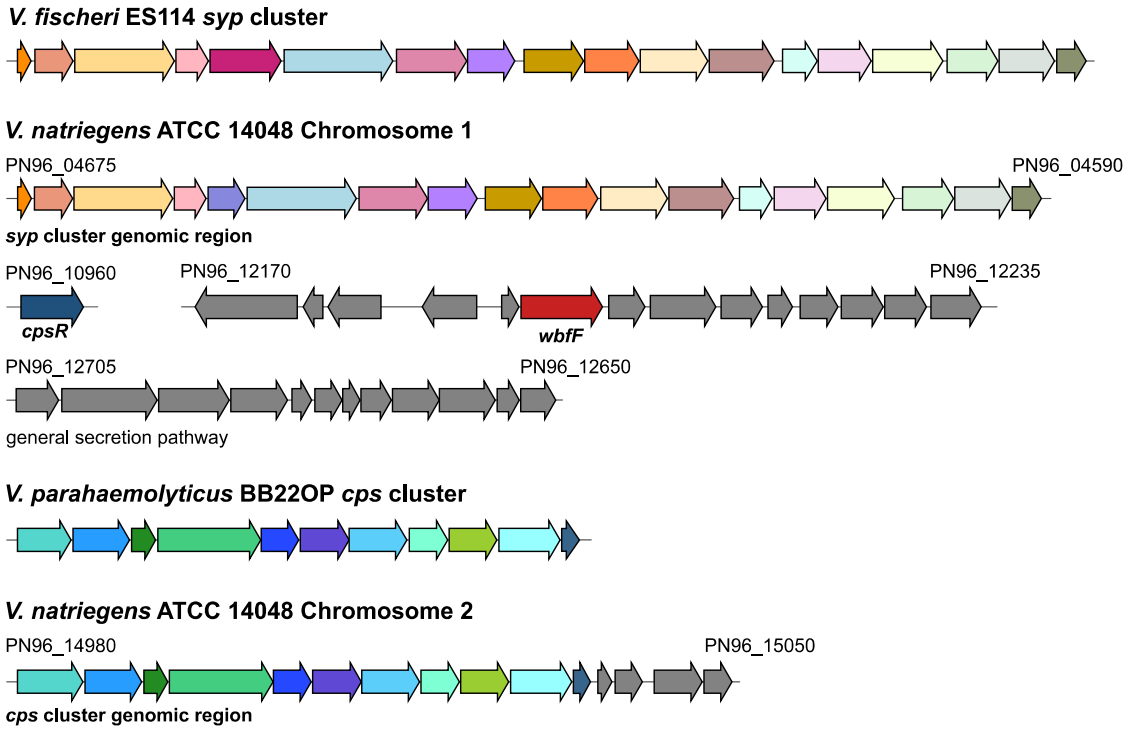

FIGURE 2 Genetic regions and genes potentially related to polysaccharide biosynthesis in *Vibrio natriegens*. Genes are represented by arrows in scale. Positions are ordered by their locations on the chromosomes and labelled with their respective gene identifiers. A detailed overview of the gene names and the corresponding locus tags is provided in Tables S2 and S3. Similarities of the *syp* and *cps* regions to published clusters of *V. fischeri* (VF_A1020-VF_A1037) (Yip et al., 2005) and *V. parahaemolyticus* (VPBB_A1276-VPBB_A1286) (Güvener & McCarter, 2003) are shown by matching colours for genes displaying over 60% sequence identity by BLASTN (Tables S2 and S3).

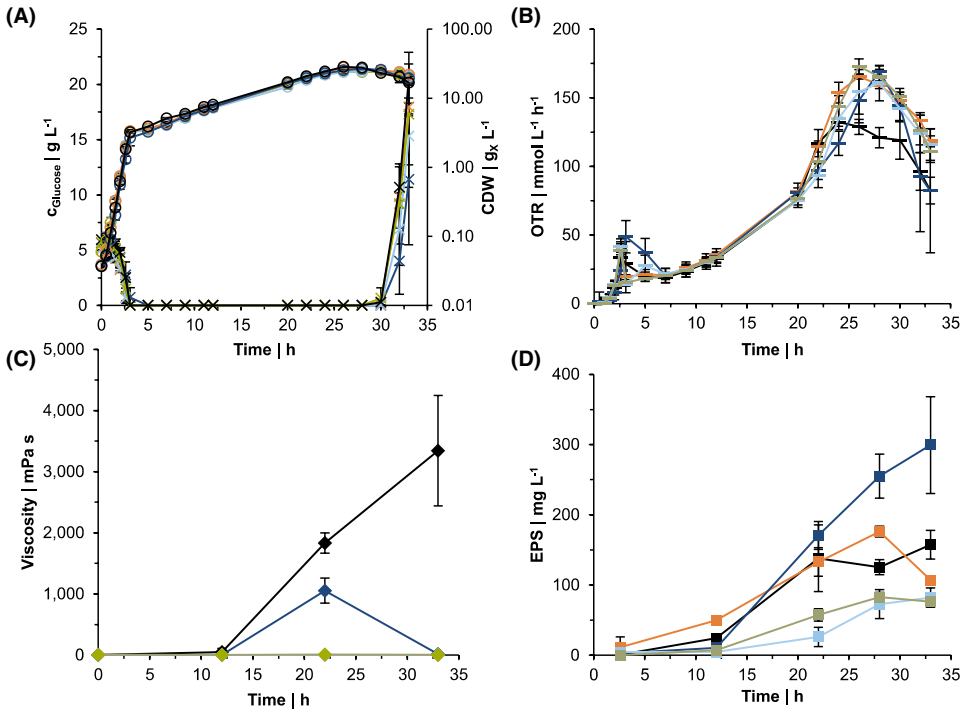

FIGURE 3 Glucose-limited fed-batch processes with *Vibrio natriegens* wt (black), *V. natriegens* Δ*cpsR* (orange), *V. natriegens* Δ*wbfF* (dark blue), *V. natriegens* Δ*cps* (light blue), and *V. natriegens* Δ*syp* Δ*cps* (green). The courses of cell dry weight (hollow circle) and glucose concentration (cross) are shown in (A). In (B), the oxygen transfer rate (bar) over time is depicted. The course of the viscosity (diamond) measured at a shear rate of 0.1 s^{-1} is shown in (C) and the EPS concentrations are pictured in (D). Results given are means and standard deviations of three independent biological replicates.

In the batch phase the strains carrying single deletions showed wt-like growth with a μ of about 1.60 h^{-1}, whereas *V. natriegens* Δ*syp* Δ*cps* exhibited a reduced μ of 1.52 ± 0.08 h^{-1}. During the fed-batch phase, all strains maintained the μ_{set} of 0.1 h^{-1} until around 26 h of fermentation and reached maximal CDWs between 24 and 28 $g_X L^{-1}$ (Table 2). Thereafter, CDW decreased in all cultivations and glucose started to accumulate in the medium. The most abundant by-products, acetate and pyruvate, were present after 24 h in all cultivations. Compared with *V. natriegens* wt, the deletion mutants produced significantly less of both metabolites (Table 2). Among the deletion mutants, *V. natriegens* Δ*cps* reached the highest acetate concentration (7.54 ± 0.9 g L^{-1}) and *V. natriegens* Δ*wbfF* the lowest one (5.18 ± 1.6 g L^{-1}). The latter produced the most pyruvate (2.67 ± 0.5 g L^{-1}), whereas *V. natriegens* Δ*syp* Δ*cps* formed the lowest amount of pyruvate (0.06 ± 0.1 g L^{-1}) among the deletion mutants (Table 2). At the end of the process, all strains produced small amounts of succinate (about 0.5 g L^{-1}) and the deletion strains additionally secreted lactate (below 1 g L^{-1}).

The oxygen transfer rate (OTR) was calculated via gas mass balance from the exhaust gas data (Figure 3B). The OTR increased steadily in all cultivations, peaked at the end of the batch phase and then further increased. After 24 h, the bioreactor with *V. natriegens* wt reached an OTR of 132 ± 3 mmol L^{-1} h^{-1} which subsequently decreased in the course of the cultivation. In contrast, for all deletion mutants the OTR further increased after 24 h and peaked between 26 and 28 h with around 170 mmol L^{-1} h^{-1} (Figure 3B; Table 2). The course of viscosity and the EPS concentration for *V. natriegens* wt increased steadily and reached a maximum of 3344 ± 903 mPa s and 157 ± 20 mg L^{-1} after 33 h, respectively (Figure 3C,D; Table 2). For *V. natriegens* Δ*wbfF*, the viscosity started to increase after 12 h, reached a maximum of 1054 ± 203 mPa s after 22 h, and decreased to 15 ± 5 mPa s after 33 h (Figure 3C; Table 2). Although the viscosity declined after 22 h, the EPS concentration in the culture broth continuously increased to a maximum of 299 ± 69 mg L^{-1} (Figure 3D). The viscosity of the reactor broth of *V. natriegens* Δ*cpsR*, *V. natriegens* Δ*cps* and *V. natriegens* Δ*syp* Δ*cps* remained between 3 ± 0 mPa s and 7 ± 1 mPa s over the process time. However, all three strains still secreted EPS reaching maximal concentrations of 176 ± 8, 82 ± 14, and 83 ± 7 mg L^{-1}, respectively (Table 2). Taken together, the deletion of *cpsR* and the *cps* cluster in *V. natriegens* wt led, compared to η_{max} of the wt, to a 1000- and 500-fold reduction of the viscosity which remained at a constant low value over the whole fermentation time. Consequently the mass transfer into the medium increased, reflected by the 25% and 22% higher OTR_{max} of *V. natriegens* Δ*cpsR*

TABLE 3 Percentage averages of the carbohydrate content between 22 and 28 h during fed-batch fermentations.

	Vibrio natriegens				
Carbohydrate content|mass % (22–28 h)	**wt**	**Δ*cpsR***	**Δ*wbfF***	**Δ*cps***	**Δ*syp* Δ*cps***
Glucose	53 ± 6	36 ± 2	64 ± 4	34 ± 10	36 ± 3
Galactose	14 ± 4	5 ± 1	22 ± 2	13 ± 2	14 ± 2
Galacturonic acid	7 ± 2	6 ± 1	5 ± 1	15 ± 3	15 ± 2
Rhamnose	12 ± 4	30 ± 2	3 ± 2	18 ± 8	16 ± 2
Fucose	4 ± 1	6 ± 1	trace	0 ± 1	0 ± 1
Glucosamine	5 ± 1	5 ± 1	3 ± 1	18 ± 11	11 ± 2
Ribose	6 ± 2	12 ± 1	4 ± 1	12 ± 5	8 ± 1

Note: Results given are means and standard deviations of three independent biological replicates. For fucose, "trace" indicates a lower value than the limit of quantification (<2 mg_{Fuc} L^{-1}).

and *V. natriegens* Δ*cps* compared with the wt. Although the rheology was significantly altered by the genetic modifications, all strains still secreted EPS into the medium under the applied conditions with *V. natriegens* Δ*cps* producing the lowest amount at 82 ± 14 mg L^{-1} (Figure 3D; Table 2).

We also analysed the carbohydrate content of the secreted EPS of *V. natriegens* wt and the deletion mutants with the HT–PMP method (Table 3). Only the EPS of the wt could be precipitated with 2-propanol. Therefore, the supernatant of the reactor broth was used for the analysis of carbohydrates. At the end of all fermentations, glucose accumulated in the medium (Figure 3A), which is an issue for the EPS quantification method. Residual glucose leads to an underestimation of the glucose content of EPS, and therefore, it is possible that EPS concentrations were even higher after 33 h for all strains. For this reason, only samples between 22 and 28 h were used for the determination of the carbohydrate composition.

The monomeric composition of the EPS differed significantly between the strains. The EPS of the strains *V. natriegens* Δ*cpsR*, *V. natriegens* Δ*cps* and *V. natriegens* Δ*syp* Δ*cps* had less glucose (around 35%) than that of *V. natriegens* wt and *V. natriegens* Δ*wbfF*. The EPS of *V. natriegens* Δ*cps* and *V. natriegens* Δ*syp* Δ*cps* showed high consistencies. The galacturonic acid, glucosamine and ribose content of both strains were elevated by a factor of 2–3 compared with the wt. The ribose content was also enhanced in the same range for *V. natriegens* Δ*cpsR* and, in addition, a strongly increased rhamnose content compared with the wt was detected. However, compared to the other strains, the EPS of *V. natriegens* Δ*cpsR* showed the lowest content of galactose (5%). In contrast, the galactose content of *V. natriegens* Δ*wbfF* was about four times higher compared with *V. natriegens* Δ*cpsR* and the least amount of rhamnose (3%) was measured. No fucose was detected in the EPS of *V. natriegens* Δ*wbfF* and the strains with EPS gene cluster deletions.

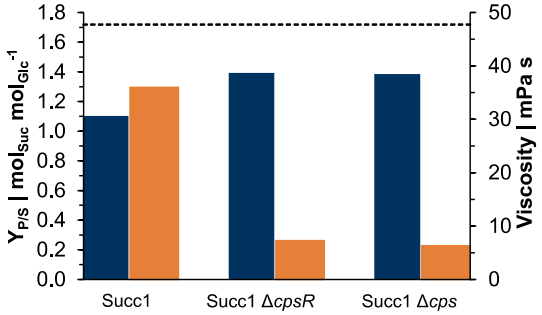

FIGURE 4 Product yield (dark blue) and final viscosity (orange) of an anaerobic resting cell approach for succinate production. Fermentations were performed with *Vibrio natriegens* Succ1, Succ1 Δ*cpsR* and Succ1 Δ*cps* ($n = 1$). The black dashed line represents the maximum theoretical yield on glucose (1.71 mol_{Suc} mol_{Glc}^{-1}). The viscosity was measured as a technical triplicate at a shear rate of 0.1 s^{-1}.

Zero-growth anaerobic succinate fermentation

Recently, we engineered *V. natriegens* for the anaerobic production of succinate from glucose and constructed the strain *V. natriegens* Succ1 (Thoma et al., 2021). During the fermentation of this strain we also observed an increased viscosity, which made it very difficult to separate the biomass from the reactor broth by centrifugation at the end of the process (data not shown). To investigate the effect of reduced EPS formation on succinate production under anaerobic conditions, we deleted the *cpsR* gene and the *cps* cluster in *V. natriegens* Succ1. Then, the recently described zero-growth process (Thoma et al., 2021) was applied for *V. natriegens* Succ1, *V. natriegens* Succ1 Δ*cpsR*, and *V. natriegens* Succ1 Δ*cps*. *V. natriegens* Succ1 reached a final titre of 45 g succinate L^{-1} within 6 h, showed a product yield $Y_{P/S}$ of 1.1 mol_{Suc} mol_{Glc}^{-1} and a volumetric productivity Q_P of 7.5 g_{Suc} L^{-1} h^{-1}. The viscosity increased from 7 to 36 mPa s at the end of cultivation (Figure 4). In

contrast, *V. natriegens* Succ1 Δ*cpsR* and *V. natriegens* Succ1 Δ*cps* showed a 5 to 6 fold decreased viscosity compared with *V. natriegens* Succ1 and the viscosity remained at the level of start of the cultivation. *V. natriegens* Succ1 Δ*cpsR* and *V. natriegens* Succ1 Δ*cps* achieved final succinate concentrations of 51 and 46 g L^{-1} with a Q_P of 8.5 g_{Suc} L^{-1} h^{-1} and Q_P of 7.7 g_{Suc} L^{-1} h^{-1}, respectively. Notably, both strains exhibited a $Y_{P/S}$ of around 1.4 mol_{Suc} mol_{Glc}^{-1}, which is 27% higher compared to the $Y_{P/S}$ of *V. natriegens* Succ1 and corresponds to 81% of the theoretical maximum (Figure 4).

DISCUSSION

In this study, a glucose-limited fed-batch process with *V. natriegens* wt was established which was accompanied by an 800-fold increase of viscosity and the secretion of EPS. The EPS was produced over the whole course of the cultivation, i.e., also in the glucose-limited fed-batch phase, which indicates that the formation of this polymer is not a result of an overflow metabolism but might rather be triggered by a regulatory mechanism such as quorum sensing in response to the cell density. Accordingly, in many *Vibrio* species EPS formation as matrix for biofilms is tightly controlled via quorum sensing (Yildiz & Visick, 2009). Quorum sensing in *V. natriegens* has not been investigated so far, however, the genome possesses essential components of the quorum sensing apparatus of *V. harveyi* such as sensor kinases (CqsS, LuxN and LuxQ) for signalling molecules (CAI-1, HAI-1 and AI-2), regulators (LuxU, LuxO and HapR) and enzymes (e.g., LuxS) for synthesis of autoinducers (Table S4; Waters & Bassler, 2005). HapR, a homologue to LuxR, is a master regulator in several *Vibrio* species which together with CpsR controls biofilm and EPS formation (Waters & Bassler, 2005). Inactivation of the transcriptional regulator CpsR in *V. natriegens* wt reduced the viscosity around 1000-fold, but did not diminish the amount of formed EPS although the composition was altered. This result indicates that CpsR is also involved in the regulation of EPS biosynthesis in *V. natriegens*. Deletion of the *cps* cluster resulted in a significantly reduced but not abolished EPS concentration in the culture broth showing that other pathways contribute to the formation of this polymer under the applied condition. In fact, our genome analysis revealed that several other genes and the *syp* cluster associated with EPS formation are present which notably amount to 0.7% of the whole genome. However, deletion of the *syp* cluster in *V. natriegens* Δ*cps* did neither further reduce the amount of EPS nor alter its composition significantly. Also, deletion of the *wbfF* gene, which has already been described to be involved in capsule biosynthesis (Dalia et al., 2017), did not reduce but doubled the amount of formed EPS indicating that other pathways contribute to EPS biosynthesis in *V. natriegens*.

The monomeric composition of EPS produced by *V. natriegens* differs from already published ones of other *Vibrio* species (Casillo et al., 2018). *V. furnissii* strain VB0S3 (Bramhachari et al., 2007) and *V. harveji* VB23 (Bramhachari & Dubey, 2006) both produce an acidic EPS with emulsifying properties and a similar monomeric structure consisting of galactose, glucose, rhamnose, fucose, mannose, ribose, arabinose and additionally xylose for the latter one. Although many EPS-producing *Vibrio* species are described in literature (Casillo et al., 2018), a structural analysis is only available for a few polymers so far. Among them are hyaluronic-like structures of HE800 produced by *V. diabolicus* (Rougeaux et al., 1999; Zanchetta et al., 2003) and the almost identical one of MO245 produced by *Vibrio* sp. isolated from bacterial mats in Moorea Island (Martin-Pastor et al., 2019). *V. alginolyticus* CNCM I-4994 produces an EPS valuable for cosmetic applications composed of D-galacturonic acid and *N*-acetyl-D-glucosamine which form a tetrasaccharide repeating unit. Alanine and serine are linked via amido linkages to D-galacturonic acid (Drouillard et al., 2015). The variability of the EPS produced by different *Vibrio* species sparks interest for potential new structures, properties and applications (Martin-Pastor et al., 2019). Therefore, future studies have to analyse the EPS structure of *V. natriegens* which is crucial to determine the physiochemical and biological properties and to evaluate the potential for industrial applications. Large-scale production of bacterial EPS typically is challenged by high costs and low yields, and most processes to date have achieved concentrations in the one- to two-digit gram range (Finore et al., 2014; García et al., 2022). So far, only limited literature about EPS production with *Vibriae* is available. Bramhachari and Dubey (2006), Bramhachari et al. (2007) reported titers of 28 mg L^{-1} and 137 mg L^{-1} of two EPS that exhibited good emulsifying properties and were secreted by *V. harveyi* VB23 and *V. furnissii* VB0S3, respectively. Higher concentrations of around 1 g L^{-1} were described for the hyaluronic-like EPS HE800 produced by *V. diabolicus* (CNCM I-1629; Delbarre-Ladrat et al., 2022) and for the EPS secreted by *V. alginolyticus* (Muralidharan & Jayachandran, 2003). Martin-Pastor et al. (2019) reported an even higher titre of 3 g L^{-1} for the EPS MO245 produced by *Vibrio* sp. from Moorea Island that shows a similar structure compared to HE800. Here, the highest titre of around 300 mg EPS L^{-1} was obtained with *V. natriegens* Δ*wbfF*, which renders this bacterium as promising host for the targeted EPS production in future studies.

Rheological measurements during the fed-batch processes were applied to monitor changes in the viscosity of the culture broth. No clear correlation was found between EPS formation and alterations

of the viscosity. *V. natriegens* wt produced EPS over the whole fermentation concomitant with a steadily increasing viscosity. In contrast, the cultures of *V. natriegens* Δ*cpsR*, *V. natriegens* Δ*cps* and *V. natriegens* Δ*syp* Δ*cps* showed no changes in viscosity although all strains still secreted EPS. However, the composition of the EPS produced by the three deletion mutants differed significantly compared with the EPS of the wt which probably altered the physicochemical properties of the polymer and its impact on the rheology of the medium. Interestingly, *V. natriegens* Δ*wbfF* steadily secreted EPS from 12 to 33 h and produced two times more compared to the wt. However, the viscosity peaked at 22 h and then declined until the end of the cultivation indicating a dynamic alteration of the EPS composition over the course of the fermentation or the secretion of a polysaccharide-degrading enzyme. It should be mentioned that not only EPS are responsible for changes in viscosity. Also other high molecular weight molecules such as proteins, nucleic acids and lipids which are produced for biofilm formation (Wingender et al., 1999) may impact the rheology of the medium. These extracellular polymeric substances were already identified in a biofilm formed by *V. natriegens* on steel in sea water (Dong et al., 2016). However, in our planktonic cultures we could not determine the presence of significant amounts of protein using the Bradford assay with bovine serum albumin as standard. Photometric analysis for nucleic acids did not provide a clear signal. Therefore, the presence of nucleic acids in the culture supernatant could not be ruled out (data not shown). Importantly, the deletion of *cpsR* and the *cps* cluster led to a constantly low viscosity reflected by an about 23% higher OTR_{max} compared to the cultivations of the wt which is highly beneficial for up- and down-stream processes of industrial biotechnology. However, although the OTR was improved, no further biomass was formed, suggesting that there is another limitation besides oxygen.

We also observed an increasing viscosity during the cultivation of *V. natriegens* Succ1 under anaerobic resting cell conditions. Consequently, we deleted *cpsR* and the *cps* cluster in this strain and observed a significantly reduced viscosity and a remarkable increase of the $Y_{P/S}$ by 25% compared to the parental strain *V. natriegens* Succ1. Although, we did not determine the EPS concentrations in these experiments, taking the increase of viscosity of the anaerobic cultivations and the EPS concentration of wt formed during the fed-batch process into account, the increase of $Y_{P/S}$ cannot solely be explained by an improved carbon availability for succinate production. EPS biosynthesis also has a substantial energy demand (Jarman & Pace, 1984; Wolfaardt et al., 1999). Therefore, the reduction of EPS formation might improve the ATP availability in *V. natriegens* Succ1 Δ*cpsR* and *V. natriegens* Succ1 Δ*cps* which can be used to drive the carboxylation towards oxaloacetate more efficiently. As discussed above, EPS biosynthesis seems to be embedded in the core regulatory regime of *V. natriegens* and therefore the introduced deletions might lead to a beneficial rerouting of the flux distribution causing the improved $Y_{P/S}$.

V. natriegens Succ1 Δ*cps* and *V. natriegens* Succ1 Δ*cpsR* show a $Y_{P/S}$ of around 1.4 mol_{Suc} mol_{Glc}^{-1}, which corresponds to 81% of the theoretical maximum, and exhibit a Q_P of 7.7 g_{Suc} $L^{-1}h^{-1}$ and Q_P of 8.5 g_{Suc} $L^{-1}h^{-1}$, respectively. Moreover, *V. natriegens* Succ1 Δ*cpsR* shows a maximum volumetric productivity of 19.4 $g\,L^{-1}h^{-1}$ which places this strain among the best-performing succinate producers available so far (Ahn et al., 2016; Dai et al., 2020; Lee et al., 2019; Yang et al., 2020).

CONCLUSION

In this study, we show that *V. natriegens* wt secretes EPS accompanied by an increasing viscosity of culture medium under aerobic and anaerobic conditions. Since EPS are a promising class of products, *V. natriegens* might represent a useful non-pathogenic host to produce native or tailored EPS with sophisticated physicochemical properties. If EPS are not the envisioned products, deletion of *cpsR* and the *cps* cluster are useful targets to reduce the amount of secreted EPS and more importantly to minimize the viscosity of the culture broth, eventually facilitating up- and downstream processing. However, besides these first insights in EPS formation, a deeper understanding of EPS biosynthesis and its regulation is required to exploit the full potential of *V. natriegens* as a host for industrial biotechnology.

AUTHOR CONTRIBUTIONS

Clarissa Schulze: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal). **Maurice Hädrich:** Formal analysis (equal); investigation (equal); methodology (equal); visualization (equal); writing – original draft (equal). **Jennifer Borger:** Investigation (equal). **Broder Rühmann:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Manuel Döring:** Formal analysis (equal); investigation (equal); methodology (equal). **Volker Sieber:** Writing – original draft (equal). **Felix Thoma:** Methodology (equal); supervision (equal). **Bastian Blombach:** Conceptualization (equal); funding acquisition (lead); project administration (lead); resources (equal); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no competing interests associated with this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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