

Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer

The Hai Pham · Nico Boon · Peter Aelterman ·
Peter Clauwaert · Liesje De Schamphelaire ·
Lynn Vanhaecke · Katrien De Maeyer · Monica Höfte ·
Willy Verstraete · Korneel Rabaey

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Abstract Previous studies revealed the abundance of *Pseudomonas* sp. in the microbial community of a microbial fuel cell (MFC). These bacteria can transfer electrons to the electrode via self-produced phenazine-based mediators. A MFC fed with acetate where several *Pseudomonas* sp. were present was found to be rich in a Gram-positive bacterium, identified as *Brevibacillus* sp. PTH1. Remarkably, MFCs operated with only the *Brevibacillus* strain in their anodes had poor electricity generation. Upon replacement of the anodic aqueous part of *Brevibacillus* containing MFCs with the cell-free anodic supernatants of MFCs operated with *Pseudomonas* sp. CMR12a, a strain producing considerable amounts of phenazine-1-carboxamide (PCN) and biosur-

factants, the electricity generation was improved significantly. Supernatants of *Pseudomonas* sp. CMR12a_Reg, a regulatory mutant lacking the ability to produce PCN, had no similar improvement effect. Purified PCN, together with rhamnolipids as biosurfactants (1 mg L⁻¹), could clearly improve electricity generation by *Brevibacillus* sp. PTH1, as well as enable this bacterium to oxidize acetate with concomitant reduction of ferric iron, supplied as goethite (FeOOH). When added alone, PCN had no observable effects on *Brevibacillus*' electron transfer. This work demonstrates that metabolites produced by *Pseudomonas* sp. enable Gram-positive bacteria to achieve extracellular electron transfer. Possibly, this bacterial interaction is a key process in the anodic electron transfer of a MFC, enabling *Brevibacillus* sp. PTH1 to achieve its dominance.

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T. H. Pham · N. Boon · P. Aelterman · P. Clauwaert ·
L. De Schamphelaire · L. Vanhaecke · W. Verstraete (✉) ·
K. Rabaey
Laboratory of Microbial Ecology and Technology (LabMET),
Ghent University,
Coupure Links 653,
9000 Ghent, Belgium
e-mail: Willy.Verstraete@UGent.be

K. Rabaey
e-mail: k.rabaey@awmc.uq.edu.au
URL: <http://labmet.UGent.be>

K. De Maeyer · M. Höfte
Laboratory of Phytopathology, Ghent University,
Coupure Links 653,
9000 Ghent, Belgium

K. Rabaey
Advanced Wastewater Management Centre,
University of Queensland,
Brisbane, QLD 4072, Australia

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Introduction

Microbial fuel cells (MFCs) are devices that convert chemical energy to electrical energy through the catalytic activity of microorganisms (Allen and Bennetto 1993; Rao et al. 1976). In a MFC, electron donors are oxidized at an anode with concomitant production of carbon dioxide, protons, and electrons. The latter are transferred to an anodic electrode (Rabaey and Verstraete 2005). Because of their unique characteristics, MFCs are expected to have a wide range of applications such as wastewater treatment and sustainable energy generation (Aelterman et al. 2006).

Several factors limit the performance of MFCs, slowing the application progress of the MFC technology in practice (Pham et al. 2006). Often mentioned are the activity of the biocatalysts, electron transfer losses both at the anodes and the cathodes, and the internal resistance (Logan et al. 2006; Rabaey and Verstraete 2005). The electron transfer between the bacterial catalysts and the anodic electrode poses a serious challenge. While highly competent microbial consortia have already been identified (Aelterman et al. 2006; Lovley 2006; Rabaey et al. 2004), only a limited amount of bacteria have thus far been studied in depth regarding their roles and mechanisms for anodic electron transfer. The existing concepts for anodic electron transfer are based on studies using mainly Gram-negative species. These include direct electron transfer via membrane associated cytochromes (Bond and Lovley 2003; Kim et al. 1999) or conductive pili or pilus-like appendages (Gorby et al. 2006; Reguera et al. 2005) and by mobile redox mediator-associated electron transfer (Rabaey and Verstraete 2005).

While in several studies, the anodic microbial communities were dominated by Gram-negative species, in a highly competent MFC system studied in our laboratory, Gram-positive *Brevibacilli* were found to dominate the community during stable operation (Aelterman et al. 2006). In a number of earlier studies, Gram-positive bacteria were prominently present in MFC systems, i.e., an *Enterococcus faecium* strain (Rabaey and Verstraete 2005) and a *Clostridium* sp. (Park et al. 2001). For the latter, some electrochemical activity could be observed, which was not the case for the *Enterococcus*. Recently, Milliken and May (2007) observed electricity generation by several *Desulfitobacterium* strains but only in combination with humic acids or their analogs. They proposed a hypothesis that Gram-positives are capable of using *external* shuttling molecules. In the reactor systems where the *Brevibacillus* species gained dominance, no humic acids were present, but instead, *Pseudomonas* species were found (Aelterman et al. 2006). As these *Pseudomonas* species are capable of producing phenazine molecules for their own proper shuttling (Rabaey and Verstraete 2005), Gram-positives may interact with *Pseudomonas* to support its energy metabolism.

In this study, we investigated the interactions between a Gram-positive *Brevibacillus* sp. isolate dominating the anodic microbial community of a MFC and a *Pseudomonas* sp. strain that produces phenazine-1-carboxamide (PCN), a phenazine compound, at elevated levels together with biosurfactants. The goal of this study was to find out whether metabolites produced by *Pseudomonas* species can actually enable Gram-positives to achieve extracellular electron transfer for a sustained energy generation in a MFC or for the reduction of iron. Based on that, the importance of this

Gram-positive–Gram-negative bacterial interaction in the electron transfer of a MFC could be conceived.

Materials and methods

Microorganisms and chemicals

Pure cultures used in this study include: (1) a Gram-positive *Brevibacillus* strain, designated PTH1, isolated from a highly competent consortium of a continuous MFC fed with acetate (Aelterman et al. 2006), (2) *Pseudomonas* sp. CMR12a originally isolated from the cocoyam rhizosphere in Cameroon, which produces PCN at elevated levels, together with biosurfactants (Perneel et al. 2007), (3) a spontaneous regulatory *Pseudomonas* mutant (CMR12a_Reg) derived from strain CMR12a with a mutation in the *gacA* gene (Heeb and Haas 2001) and thus lacking the ability to produce secondary metabolites (including PCN, as well as biosurfactants). The strains were deposited in the Belgian Co-ordinated Collections of Micro-Organisms/Laboratorium voor Microbiologie (LMG) bacteria collection (accession numbers LMG 23887 for *Brevibacillus* sp. PTH1, LMG 24333 for *Pseudomonas* sp. CMR12a, and LMG 23889 for *Pseudomonas* sp. CMR12a_Reg). A biosurfactant solution, the PRO1 (De Jonghe et al. 2005), containing 25% rhamnolipids was supplied by Plantsupport (The Netherlands).

Bacterial isolation

Bacteria were isolated from a mixed culture sample taken from an MFC fed with acetate by applying conventional methods. Media used to cultivate isolates include LB (Luria–Bertani with composition per liter: tryptone 10 g, yeast extract 5 g, NaCl 10 g and distilled water), R₂A (from Bacto R2A agar), and M9 (with composition per liter: 6 g Na₂HPO₄, 1 g NH₄Cl, 0.5 g NaCl, 0.2465 g MgSO₄·7H₂O, 3 g KH₂PO₄, 14.7 g CaCl₂, and distilled water). When used for isolation under anoxic conditions, the M9 medium was supplemented with sodium or potassium nitrate or sulfate (to a concentration of 20 mM) to provide sufficient alternative electron acceptors. The anoxic isolation dishes were kept in anaerobic jars; anaerobic conditions were established using catalysts (Oxoid, UK). Gram staining was carried out following the standard procedure (Madigan et al. 2004).

Denaturing gradient gel electrophoresis

Total deoxyribonucleic acid (DNA) of the mixed culture and of the isolates was extracted using standard methods (Boon et al. 2000). 16S ribosomal ribonucleic acid (rRNA)

gene fragments were amplified with the primers PRBA 338fGC and P518r (Muyzer et al. 1993) and analyzed by denaturing gradient gel electrophoresis (DGGE) with a denaturing gradient ranging from 45 to 60% (Boon et al. 2002). All the isolates were analyzed, and the isolate representing the dominant species was determined based on the migration correspondence of its DGGE bands to strong bands on the DGGE pattern of the mixed culture.

16S rRNA gene amplification, sequencing, and BLAST analysis

16S rRNA gene sequences of the isolates were amplified with the primers P63F and R1378r (Ovreas et al. 1997). DNA sequencing of the sequences obtained was carried out by ITT Biotech Bioservice (Bielefeld, Germany). The analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the Basic Local Alignment Search Tool (BLAST) server of the National Center for Biotechnology Information using the BLAST algorithm (Altschul et al. 1997).

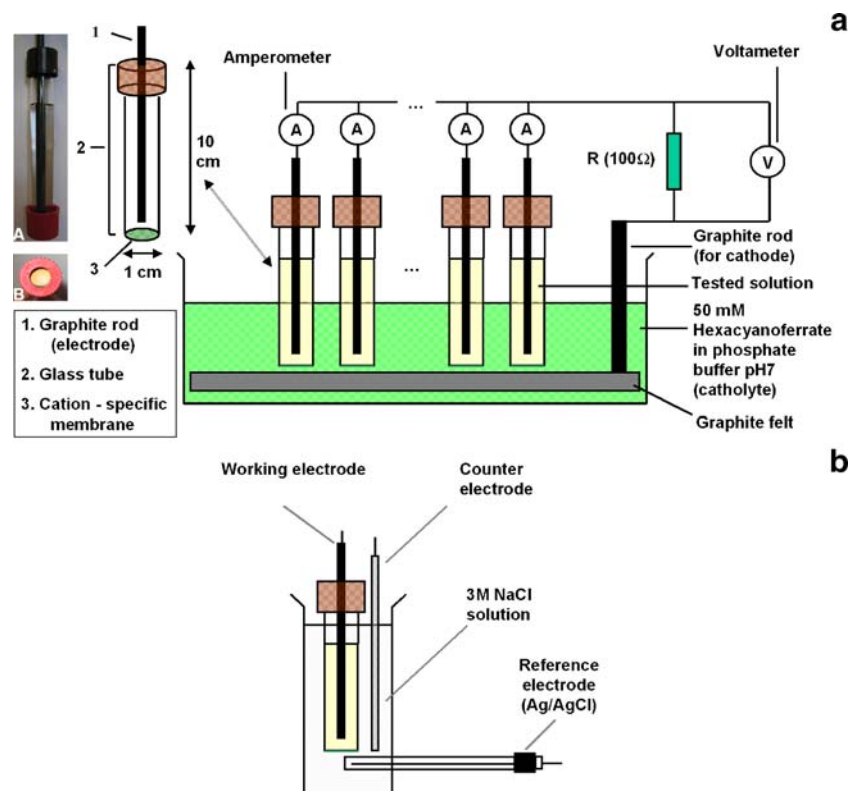
Test tube MFC system

A system for MFC-based cultivation of single strains and analysis of their electrochemical activity was developed (Fig. 1a). In the system, the cell suspension of a culture or

a solution of interest was injected into a sterile glass tube with 10 mL working volume (referred to as test tube MFC, TTMFC). The bottom end of the tube was sealed with a 1-cm² circular cation exchange membrane (Ultrex CMI7000, Membranes International, USA) using a superglue (SUPERCOL, BricoBi, Belgium) before it was capped tightly with an open-topped cap. The top end was also capped tightly and supported a 9.5-cm-long part of a 5-mm-diameter graphite rod (Morgan, Belgium), submerged into the cell suspension. After capping, the inside of the tube was completely isolated from the outside. Before the injection of the cell suspension, the tube was sterilized by submersion in ethanol (70%) for 1 h for three times and subsequently dried under UV light in a laminar flow.

The membrane ends of the TTMFCs were submerged into a hexacyanoferrate catholyte (100 mM phosphate buffered 50 mM potassium hexacyanoferrate solution) and contacted a graphite-felt electrode serving as the cathode. The graphite rods of the tubes were connected through wires to a 100- Ω resistor that closed the circuit with the cathode. The system, in which each tube was actually a half cell, thus became a multi-MFC system that could be operated for days or weeks. The current generated by each tube (an MFC), as the signal to evaluate the electrochemical activity, was regularly measured using an amperometer. All the TTMFCs in this research, unless otherwise stated, were operated with bacteria in LB broth as the basic medium.

Fig. 1 Test tube microbial fuel cell (TTMFC) system (a), cyclic voltammetry setup (b). Images A and B (letters in white) showing the real side view and bottom view of such a test tube, respectively



In some experiments, separation of bacterial cells and the anodic aqueous part in each TTMFC was needed, and this was done by anaerobically transferring the anodic suspension to a sterile plastic tube and centrifuging it at $2,300\times g$ for 15 min. Bacterial cells of interest were washed three times with sterile phosphate buffer (100 mM, pH 7) before being used further. If an anodic supernatant was to be used to replace another, it was subsequently filter sterilized using a 0.22- μm syringe filter unit (Millex, USA) before being mixed with the bacterial cells of interest in a new TTMFC.

To ensure that no contamination interfered with the experimental results, all the manipulations with bacterial cell suspensions (except for centrifugation) were carried out under sterile conditions in a laminar flow. In addition, the axenicity of the bacterial cells was verified using light microscopy. The filtered supernatants were plated on LB agar to verify sterility. Only experimental data obtained with no contamination were considered valid and used for analysis.

Cyclic voltammetry

To ensure sterile working conditions, for the voltammetry analysis, a half-cell TTMFC (as described above) containing the cell suspension or the solution of interest was removed from the system and carefully washed with water to ensure that no hexacyanoferrate remained on its surface. Subsequently, the tube was submerged into a 50-mL vial containing 3 M NaCl solution, together with a platinum counter electrode and a Ag/AgCl (assumed +197 mV vs standard hydrogen electrode [SHE]) reference electrode (BASi, Warwickshire, UK; Fig. 1b). The graphite rod (anode) of the tube was employed as the working electrode. The cyclic voltammetry was performed as described previously (Rabaey et al. 2005b). A Bi Stat potentiostat (Princeton Applied Research, Claix, France), branched to a personal computer PC was used and operated with EC Lab software (Princeton Applied Research, USA), at scan rates of 5 or 10 mV s^{-1} depending on experimental cases and in the potential range of -0.6 or -0.4 to 0.8 or 1 V (vs SHE). For each sample, scanning was repeated three times. Appearance of oxidation/reduction peaks in a cyclic voltammogram indicates a signal of electrochemical activity. Controls were performed to verify the accuracy of the voltammetry measurements.

To check the reliability of the set up, cyclic voltammetry analysis of hexacyanoferrate was done both using this setup and in a conventional way (all the electrodes in one test tube). Compared to those obtained with a conventional setup, in cyclic voltammograms obtained from this new setup, the redox potentials of the peaks were slightly offset, but the appearance of the peaks was not altered. Therefore,

the setup was considered applicable for electrochemical activity analysis.

Extraction, analysis, and purification of phenazine-1-carboxamide

Pseudomonas sp. CMR12a was grown in 1 L of King B medium (King et al. 1954). After 5 days of incubation on a shaker at room temperature, the culture suspension was centrifuged, and the supernatant was mixed with an equal volume of chloroform. The mixture was shaken several times and left for 1 h for the phases to be separated. The chloroform phase was recovered and subsequently mixed with an equal volume of 0.1 N NaOH solution to purify the compound(s) obtained. Finally, the chloroform phase was again taken and dried. The remainder was dissolved in methanol before being analyzed by high-performance liquid chromatography (HPLC).

The HPLC analysis was performed as described by Fernandez and Pizarro (1997) and Mavrodi et al. (2001) on a Dionex HPLC system (Sunnyvale, CA) comprising an autosampler ASI 100, a pump series P580, and a STH585 column oven, coupled to a UVD340S UV/VIS detector. Mass spectra were recorded by coupling the Dionex HPLC system to an Agilent Single Quad MS detector equipped with an electrospray ionization source, operated in the positive ion mode (Agilent Technologies, Diegem, Belgium). A 150×4.6 -mm inner diameter, 4 μm , Genesis C18 column (Jones Chromatography, UK) was used. The temperature was set at 25°C , and the flow rate was maintained at 1 mL min^{-1} . Solvents were water/trifluoroacetic acid (100:0.04, v/v; solvent A) and acetonitrile/water/trifluoroacetic acid (90:10:0.04, v/v/v; solvent B). Elution was as follows: Solvent A was maintained for 15 min and then changed to 90% A and 10% B. This mixture was applied for 10 min. A linear gradient to 70% A and 30% B in 15 min was then applied. These conditions were maintained for 5 min (until 45 min from the onset). Finally, solvent composition was changed to 64% A and 36% B and maintained until the end (65 min from the starting time). UV absorbance was monitored at 250 and 390 nm with a bandwidth of 4 nm in all cases. Data were collected and peaks integrated using the Chromeleon chromatography manager software (Dionex).

Liquid chromatograms of the chloroform extract obtained from the extraction done for the liquid culture of strain CMR12a at 250 nm as well as 390 nm both showed a strong peak with a retention time of about 43.1 min. The peak was found to correspond with the presence of PCN, as the corresponding peak in the mass spectra represents a compound with a molecular weight (MW) of 223 (m/z value = $M + 1 = 224$), which is that of PCN. In addition, the methanol solution dissolving the extract had

maximal absorbance peaks at wavelengths of about 247 and 364 nm, which are also reported as the peak maxima of the UV spectra of PCN under these conditions (Mavrodi et al. 2001). HPLC and mass spectrum analysis showed that the chloroform extract contained a large amount of mainly PCN (MW=223) and a little PCA (MW=224), its precursor. The other components were negligible. This analysis information was used for the preparative separation (purification) of PCN.

The preparative separation of PCN was performed on an Agilent preparative HPLC system (Agilent Technologies) comprising a G1361A pump system and a Agilent 1100 series fraction collector, coupled to a model G1314A UV/VIS detector. Chromatographic separation was achieved using a Zorbax Extend C18 column (21.2×150 mm inner diameter, 5 µm) obtained from Agilent Technologies. Elution was carried out using the same procedure as stated above; the flow rate was 20 mL min⁻¹. Absorbance was monitored at 390 nm.

Other analytical techniques

The presence of biosurfactants in the supernatants of interest was detected based on the spreading ability of the supernatants on parafilm, following the drop collapsing method (Jain et al. 1991). Optical density of the cell suspensions was measured using a UV and visible light spectrophotometer (Uvikon 932, Kontron Instruments). Chemical oxygen demand (COD) measurements were done according to the dichromate method (Greenberg et al. 1992). Measurement of ferrous ion was done applying the ferrozine method (Lovley and Phillips 1986; Park et al. 2001). Acetate was measured using gas chromatography as previously described (Rabaey et al. 2005b).

All experiments were carried out in triplicate. Statistical errors were calculated following standard methods.

Results

Isolation and identification of species dominating the microbial community of an MFC fed with acetate

A Gram-positive bacterium with rod-shaped cells, designated as PTH1, was isolated from the anodic compartment of an MFC fed with acetate. The isolate was identified to be one of the *Brevibacillus* species dominating the anodic microbial community of the MFC, based on DGGE analysis and 16S rRNA gene sequence analysis (GenBank accession number of the sequence: EU170241). Furthermore, applying the same procedure, also eight *Pseudomonas* sp. strains were found (see [Electronic supplementary material](#)).

Electrochemical activity of the axenic cultures in the study

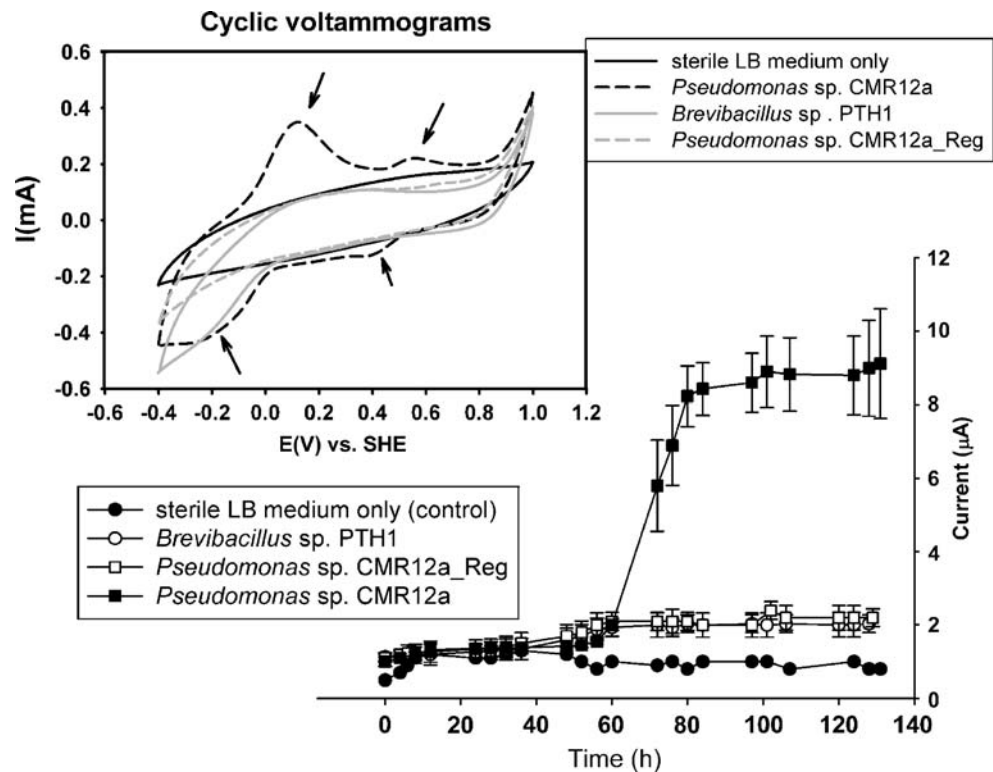
Axenic cultures of the strains of interest were inoculated and tested for their electrochemical activity in the TTMFC system. This was combined with cyclic voltammetry analysis. Sustained current generation and the appearance of oxidation/reduction peaks in cyclic voltammograms (Rabaey et al. 2004) were considered as indicators of electrochemical activity.

According to that, from the results depicted in Fig. 2, it can be seen that *Brevibacillus* sp. PTH1 was not or barely electrochemically active. *Pseudomonas* sp. CMR12a, the strain known to be capable of producing PCN at elevated levels and biosurfactants, expressed electrochemical activity and generated currents up to 8–10 µA (Fig. 2). In the cyclic voltammogram of *Pseudomonas* sp. CMR12a, two pairs of peaks can be distinguished: one with lower intensity, corresponding to a component with a midpoint potential of about +450 mV vs SHE, and the other with much stronger intensity corresponding to a component with a midpoint potential of about -150 mV vs SHE (Fig. 2). The regulatory mutant of CMR12a, CMR12a_Reg, which is deficient in producing secondary metabolites, did not show any electrochemical activity (Fig. 2).

Interactions of *Brevibacillus* sp. PTH1 with *Pseudomonas* sp. CMR12a

When TTMFCs that had been operated with *Pseudomonas* sp. CMR12a for 1 week were supplemented with the bacterial cells harvested from TTMFCs operated with *Brevibacillus* sp. PTH1, their currents increased from 8–10 to about 12–15 µA (data not shown). To investigate if the anodic aqueous part of the TTMFCs operated with the *Pseudomonas* strain can improve the current generation by *Brevibacillus* sp. PTH1, the anodic supernatant of a TTMFC with *Brevibacillus* sp. PTH1 was replaced with that of a TTMFC operated with *Pseudomonas* sp. CMR12a. Upon the replacement, the currents generated by the TTMFCs operated with PTH1 increased from 2 up to 13 µA and subsequently to a stable level of about 9–10 µA (Fig. 3). Cyclic voltammograms of the half-cell tubes taken from the TTMFCs operated with cells of *Brevibacillus* sp. PTH1, and the replaced aqueous part had peaks appearing (Fig. 3, cyclic voltammogram B), becoming similar to those in the experimental cases with axenic *Pseudomonas* sp. CMR12a cultures (Fig. 2). Removing the bacterial cells from these TTMFCs resulted in a decrease in current generation and lower peak intensity in the cyclic voltammograms of their half-cell tubes, although the peaks could still be discerned clearly (Fig. 3, cyclic voltammogram C). The same experiments were done to investigate interactions between *Brevibacillus* sp. PTH1 and the mutant *Pseudomo-*

Fig. 2 Patterns of current production by the test tube MFCs operated with the axenic cultures (*down right*) and the typical cyclic voltammograms of their half cell tubes (*upper left*). Arrows in the cyclic voltammograms indicate peaks



nas sp. CMR12a_Reg. As shown in Fig. 3 (Experiment 3), the electrochemical activity of PTH1 was not significantly enhanced by the supernatant of the TTMFCs operated with CMR12a_Reg.

Based on the results obtained, we hypothesized that one or more components in the *Pseudomonas* sp. CMR12a supernatant could function as electron shuttles for *Brevibacillus* sp. PTH1 to use, and PCN was very likely the major one among them because it is the main metabolite produced by this *Pseudomonas*. Therefore, subsequent experiments were aimed at investigating the role of PCN in the electron transfer by *Brevibacillus* sp. PTH1.

PCN, rhamnolipids, and the electricity generation by *Brevibacillus* sp. PTH1

Supplementing purified PCN (to a final concentration of 50 mg L⁻¹) directly to the anodic content of each of TTMFCs operated with *Brevibacillus* sp. PTH1 did not lead to an increase in current generation by the TTMFCs (Fig. 4). To investigate if biosurfactants, which strain CMR12a also produced, could have some synergistic effect with PCN, the anodic content of the TTMFCs was supplemented with daily increasing amounts of a sterile nonionic surfactant solution containing 25% rhamnolipids. As can be seen in Fig. 4, adding rhamnolipids up to the concentration of 1 mg L⁻¹ led to significant increases of the current generated by the TTMFCs that contained PCN.

The increased currents were decreased if the concentrations of rhamnolipids are higher than 1 mg L⁻¹ (Fig. 4). While the currents changed, the density of the bacterial cells did not change significantly (data not shown). Surfactants without PCN have no observable effect on the current generation.

Cyclic voltammograms of pure PCN (the inlet in Fig. 5) showed substantial peaks similar to those of axenic CMR12a cultures (Fig. 2) and of the tubes operated with bacterial cells of *Brevibacillus* sp. PTH1 and the cell-free anodic supernatant of TTMFCs operated with CMR12a (Fig. 3). The height of the peaks when only PCN was in the medium (LB) was lower than that in the case of the mixture of PCN and rhamnolipids.

Effect of increasing concentrations of PCN on the electricity generation by *Brevibacillus* sp. PTH1

To investigate if PCN can really function as an electron mediator, the concentrations of purified PCN were increased daily in the anode of TTMFCs operated with *Brevibacillus* sp. PTH1 and already containing rhamnolipids (1 mg L⁻¹). Adding only rhamnolipids did not result in any increase in the currents (Fig. 5). As can be seen in Fig. 5, a clear dose response of the currents generated could be observed when the concentration of PCN reached to about 10 mg L⁻¹. During the development of the currents, the cell density did not change much (data not shown).

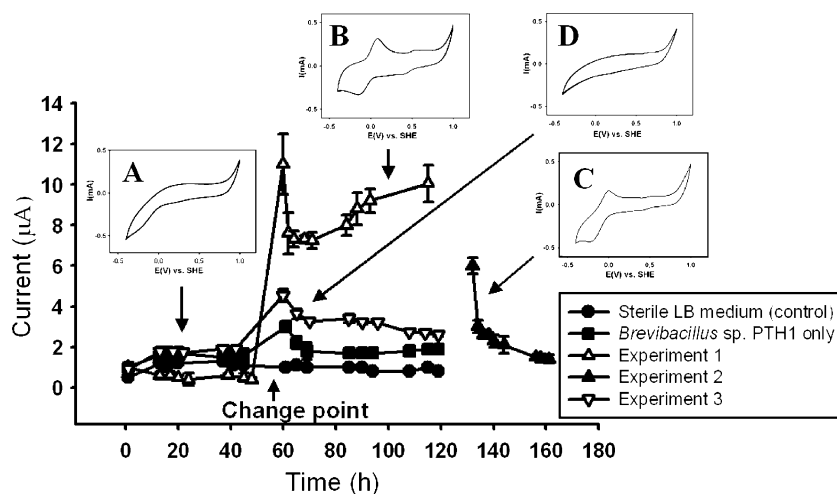


Fig. 3 Patterns of current generation of the TTMFCs and corresponding cyclic voltammograms of their half cell tubes in the experiments testing the interactions of *Brevibacillus* sp. PTH1 with the electrochemically active *Pseudomonas* sp. CMR12a and its regulatory mutant CMR12a_Reg. *Experiment 1*: Before the change point, the TTMFCs were operated with *Brevibacillus* sp. PTH1 (A); after the change point, the TTMFCs were operated with cells of *Brevibacillus* sp. PTH1 and the cell-free anodic supernatants of the TTMFCs that had been operated with *Pseudomonas* sp. CMR12a for 1 week (B); *Experiment 2*: new TTMFCs operated with only the cell-

free anodic supernatants of the TTMFCs in case B (C); *Experiment 3*: before the change point, the TTMFCs were operated with *Brevibacillus* sp. PTH1 (A); after the change point, the TTMFCs were operated with cells of *Brevibacillus* sp. PTH1 and the cell-free anodic supernatants of the TTMFCs that had been operated with *Pseudomonas* sp. CMR12a_Reg for 1 week (D). Cyclic voltammograms are labeled corresponding to experimental cases (A, B, C...). For the TTMFCs operated with *Brevibacillus* sp. PTH1 only, at the change point, the supernatants were replaced by fresh LB medium

Effect of PCN, rhamnolipids, on the use of acetate as electron donor and FeOOH as electron acceptor by *Brevibacillus* sp. PTH1

Cell cultures of *Brevibacillus* sp. PTH1 were cultivated anaerobically in serum flasks containing M9 medium with only acetate (1 g L^{-1}) as the electron donor. Adding PCN (to 10 mg L^{-1}) together with rhamnolipids (1 mg L^{-1}) to the cultivated medium after 1 day of cultivation resulted in a significant decrease in acetate concentration in the medium (Fig. 6a). Further addition of FeOOH (to 2 g L^{-1}) did not lead to noticeable changes of this decrease, but increasing amounts of ferrous ion in the medium could be observed (Fig. 6b). If FeOOH was added at day 1 instead of PCN and rhamnolipids, the decrease in acetate concentration was not significant (Fig. 6a), and the concentration of ferrous ion did not change much (Fig. 6b). However, at day 4, if PCN and rhamnolipid were further added to these FeOOH containing flasks, acetate concentration decreased, and the concentration of ferrous ion rose (Fig. 6b). Such phenomena could not be observed with the flasks containing no bacteria (control).

Discussion

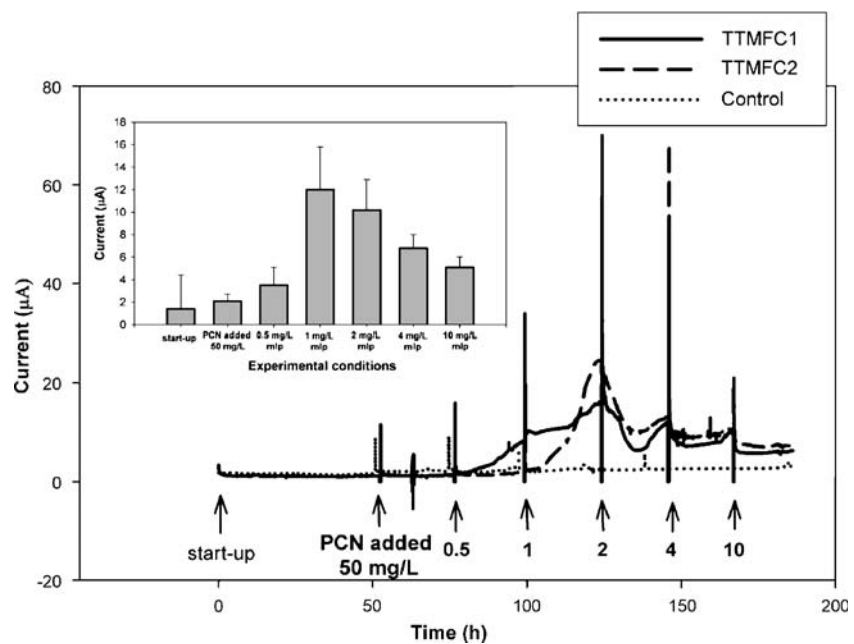
While the construction of conventional MFCs is expensive and labor intensive, the TTMFCs enable us to study

individual as well as interactive activity of a multiplicity of axenic cultures. It should be noted that the emphasis of this work was on microbial interaction, and aspects such as power efficiency or COD removal were not considered. However, overall, mechanistic principles that are true for this system can be also applicable in larger systems.

Electrochemical activity of the Gram-positive *Brevibacillus* sp. PTH1 and its interactions with *Pseudomonas* sp. CMR12a in a MFC

Beside one study by Park et al. (2001), no electrochemically active Gram-positives have been described thus far. It is a query how these bacteria can interact with electrodes while they have a rigid cell wall with peptidoglycan layers. In a recent study by Milliken and May (2007), Gram-positives that were not electrochemically active were triggered to produce current by the addition of humic acids or their analogs. However, there is little known about the true mechanism through which Gram-positive bacteria demonstrate electrochemical activity. In this study, the Gram-positive *Brevibacillus* sp. PTH1 was poorly electrochemically active, and it could only achieve electron transfer to an electrode once being in contact with the aqueous environment created by an electrochemically active *Pseudomonas* strain—the CMR12a (Figs. 2 and 3). It has been shown before that *Pseudomonas* species have a strong capability of producing metabolites functioning as

Fig. 4 Current patterns of two TTMFCs operated with *Brevibacillus* sp. PTH1 in the experiments with purified PCN and increasing amounts of rhamnolipids. At 50 h (after 2 days of operation), purified PCN was added into the anodic content of each TTMFC at the concentration of 50 mg L⁻¹. Subsequently, rhamnolipids were daily added to the corresponding concentrations indicated by the numbers under arrows. *rnlp* Rhamnolipids, *control* without rhamnolipids addition. The *inlet* shows the relationship between the average current generated and the amount of rhamnolipids present in the anodic content



soluble mediators for extracellular electron transfer in general (Hernandez and Newman 2001) and anodic electron transfer in particular (Rabaey et al. 2004). Therefore, it appears most likely that some metabolites produced by *Pseudomonas* sp. CMR12a either can serve as soluble electron shuttles for the Gram-positive *Brevibacil-*

lus to use or that they at least invoke extracellular electron transfer by *Brevibacillus*. The effect of PCN, a metabolite produced by *Pseudomonas* sp. CMR12a, on the electron transfer by *Brevibacillus* sp. PTH1 was shown, in this study, as a good demonstration for this hypothesis. Cyclic voltammetry results showed that there might be other

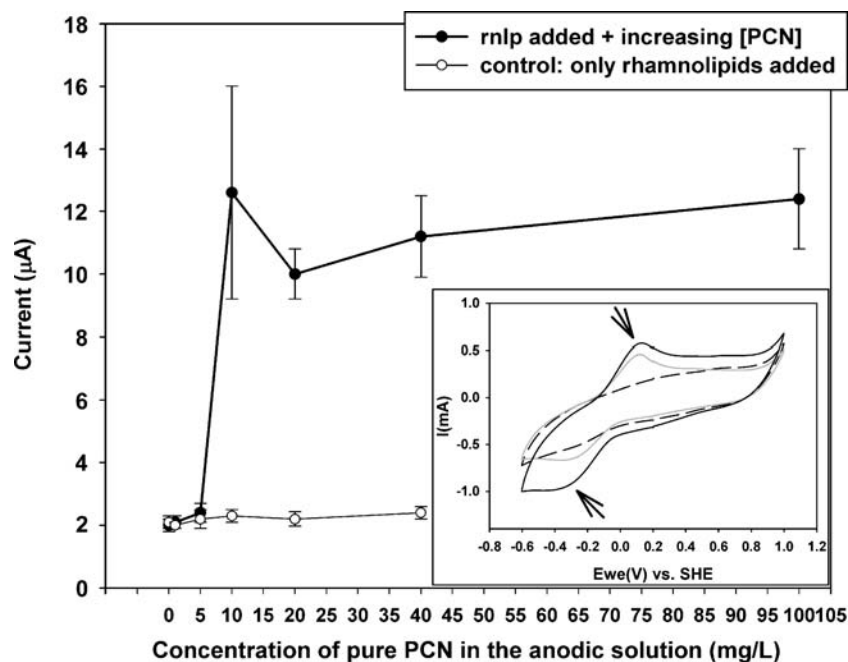
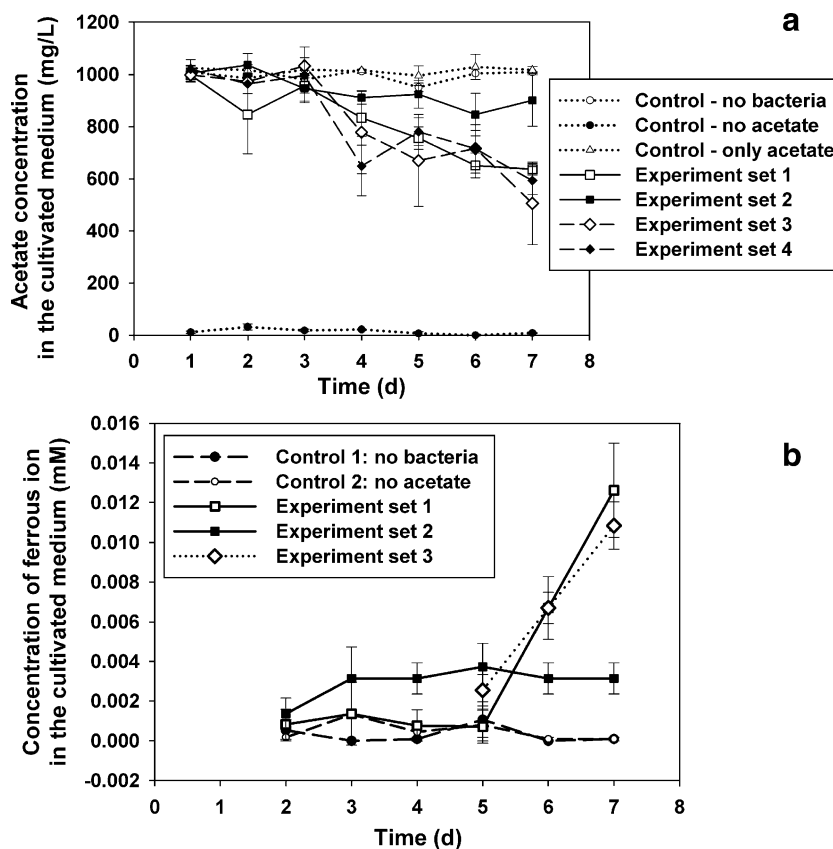


Fig. 5 The relationship between the amount of PCN in the anodic content and the currents of TTMFCs operated with *Brevibacillus* sp. PTH1. In every case, the anodic content of the TTMFCs already contained 1 mg L⁻¹ of rhamnolipids. The *inlet* shows typical cyclic

voltammograms of sterile LB medium (*dashed line*), purified PCN in sterile LB medium (*light line*), and purified PCN in sterile LB medium containing 1 mg L⁻¹ of rhamnolipids (*solid line*). Arrows indicate peaks

Fig. 6 Changes of the concentration of acetate (a) and ferrous ion (b) in different flasks inoculated anaerobically with *Brevibacillus* sp. PTH1 in M9 medium containing 1 g L⁻¹ of acetate. *Experiment set 1*: After day 1: FeOOH added (to 2 g L⁻¹), after day 4: PCN and rhamnolipid added (to 10 and 1 mg L⁻¹, respectively); *Experiment set 2*: only FeOOH added after day 1; *Experiment set 3*: after day 1: PCN and rhamnolipid added (to 10 and 1 mg L⁻¹, respectively), after day 4: FeOOH added (to 2 g L⁻¹); *Experiment set 4*: only PCN and rhamnolipid added after day 1



metabolite(s) of interest (corresponding to the peaks observed at the redox potential of about +450 mV), but we have thus far been unable to identify these.

PCN and the electrochemical activity of *Pseudomonas* sp. CMR12a

It was shown from the results, especially from cyclic voltammograms, that *Pseudomonas* sp. CMR12a is electrochemically active and its electrochemical activity is closely related to PCN, the main redox-active metabolite that it produces. Experimental results with the regulatory mutant CMR12a_Reg, defective in the ability to produce PCN and other secondary metabolites, also suggested that the metabolites are crucial for the electrochemical activity of *Pseudomonas* sp. CMR12a. It has been shown before that phenazine compounds, including PCN, produced by *Pseudomonas* spp. can function as electron shuttles enabling these bacteria to transfer electrons to the anode in a MFC (Rabaey et al. 2005a).

Can PCN shuttle electrons for *Brevibacillus* sp. PTH1?

The dose response of the currents generated and the unaffected cell density of PTH1 upon increasing the concen-

tration of purified PCN in the rhamnolipids-containing anodic content of the TTMFCs with PTH1 (Fig. 5) strongly suggested that PCN can function as an electron mediator for *Brevibacillus* sp. PTH1 to transfer electrons, provided rhamnolipids are simultaneously present in low concentrations. The significant decrease in the acetate concentration in the medium of serum flasks supplemented with PCN and rhamnolipids indicate that *Brevibacillus* sp. PTH1 can reduce PCN to oxidize the electron donor, although the reduction in the PCN itself could not be measured in the used medium. The increase in the ferrous ion concentration whenever FeOOH was present in the medium together with the PCN/rhamnolipids mixture shows that PCN, in turn, can transfer electrons to FeOOH. However, it should be noted that the increased concentration of ferrous ion (at the μM level) was not significant, although the increasing trend is apparent (data for all three serum flasks in the experiment are highly consistent). This lower activity, compared to current generation, can be expected based on the fact that FeOOH is not a favorable electron acceptor in comparison to an electrode. As reported by Hernandez et al. (2004), the production of Fe (II) as the result of the reduction of ferric ion by the well-known metal reducer *S. oneidensis* MR1 was also at the μM level and could also be promoted by the mediation of PCN.

One noticeable point is that PCN could only improve the electrochemical activity of the Gram-positive *Brevibacillus* sp. PTH1 if the biosurfactant rhamnolipids were also present. Rhamnolipids alone had no observable effect on the electrochemical activity of PTH1. In other words, possibly, PCN and rhamnolipids have a synergistic effect on the electrochemical activity of *Brevibacillus* sp. PTH1. The most reasonable explanation for this is that rhamnolipids, which are produced by many *Pseudomonas* spp., improve the solubility and hence the availability of PCN, helping it penetrate and really function as an electron shuttle. It has been described that rhamnolipids can solubilize rather hydrophobic phenazines (at normal pH levels) within micelle structures (Al-Tahhan et al. 2000). Indeed, we observed that when being mixed with the LB medium, part of the PCN particles apparently remained in the solid state, but they dissolved more if rhamnolipids were added into the medium. Furthermore, drops of the supernatant of the TTMFCs operated with *Brevibacillus* sp. PTH1 hardly collapsed on parafilm (indicating the absence of biosurfactants [Jain et al. 1991]), unlike those of the TTMFCs operated with *Pseudomonas* sp. CMR12a (data not shown).

In our study, the decrease in the current generation by *Brevibacillus* sp. PTH1 when the concentration of rhamnolipids was higher than 1 mg L^{-1} showed that too much rhamnolipids is harmful for the bacteria. As observed by Boles et al. (2005), rhamnolipids can cause biofilm detachment. Possibly, rhamnolipids are harmful to *Brevibacillus* sp. PTH1 in some similar way.

The overall concept that the Gram-positive *Brevibacilli* use electron shuttles produced by other bacteria is intriguing. The interaction of this species with *Pseudomonas* spp., more specifically with secondary metabolites produced by *Pseudomonas* spp., is proposed as the most reasonable explanation for their presence and dominance in the microbial community of the MFCs fed with acetate. Moreover, this bacterial interaction could be a base to develop approaches to improve the anodic electron transfer in a MFC. Nevertheless, the role of other factors such as quorum-sensing molecules and/or other mediators should not be underestimated and need to be focused on in further studies.

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