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Microbial Phenazine Production Enhances Electron Transfer in Biofuel Cells

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High-rate electron transfer toward an anode in microbial fuel cells (MFCs) has thus far not been described for bacteria-producing soluble redox mediators. To study the mechanism of electron transfer, we used a MFC isolate, *Pseudomonas aeruginosa* strain KRP1. Bacterial electron transfer toward the MFC anode was enabled through pyocyanin and phenazine-1-carboxamide. The presence of the anode stimulated pyocyanin production. Mutant strains, deficient in the synthesis of pyocyanin and phenazine-1-carboxamide, were unable to achieve substantial electron transfer and reached only 5% of the wild type's power output. Upon pyocyanin addition, the power output was restored to 50%. Pyocyanin was not only used by *P. aeruginosa* to improve electron transfer but as well enhanced electron transfer by other bacterial species. The finding that one bacterium can produce electron shuttles, which can be used also by other bacteria, to enhance electron-transfer rate and growth, has not been shown before. These findings have considerable implications with respect to the power output attainable in MFCs.

Introduction

Microbial fuel cells (MFCs) convert the metabolic energy which bacteria obtain from their electron donor directly into electricity (*1*). The electrons flow from the bacteria toward an anode and further through an external circuit. At the cathode, the electrons are used to convert oxygen into water, closing the cycle. Several types of MFCs have recently gained increased attention: (i) sediment MFCs, in which the potential difference between a sediment and the liquid phase above is used to generate power (*2*), (ii) photoheterotrophic MFCs, in which (alternating) light and carbon substrate are provided to, e.g., cyanobacteria which in turn can use an electrode as electron acceptor (*3*), and (iii) heterotrophic "dark" reactor MFCs, in which (facultative) anaerobic bacteria use carbon substrates to generate reducing power (*4*). Both for discrete substrates such as glucose (*5*) and diverse substrates such as wastewater (*6*), promising results have been obtained.

One of the main bottlenecks in MFCs is the electron transfer from the bacteria to the anode (*7*). Either oxidizing a compound at the anode surface or reducing a compound at the bacterial surface or in the bacterial interior surface can require certain energy to activate the oxidation reaction. This causes a transfer resistance and hence potential losses between the bacteria and the electrodes, which are generally called overpotentials (*8*). Technological or bacterial adaptations can alleviate these high overpotentials to use the surface more efficiently as electron acceptor.

Technological improvement can be achieved through an increase of the specific surface of the electrode to enhance contact between the bacteria and the electrode. This increase of the electrode is only efficient to a limited extent, since bacteria form biofilms clogging small pores and the feed needs to flow freely through the electrode matrix. Also the replacement or omission of the proton exchange membrane can increase the power output (*9*).

Bacterial optimization encompasses either the expression of membrane-associated or mobile, soluble redox mediators. A redox mediator is a molecule that functions as an electron shuttle between bacteria and an electron acceptor. It can be oxidized more rapidly at the electrode, which causes a decrease in overpotential losses. Efficient electron transfer through membrane-associated, mediating complexes from bacteria attached to the electrode has been reported with *Rhodoferax ferrireducens* (*10*). Expression of sterically accessible membrane proteins increases the electron-transfer rate of the bacteria toward the extracellular, nonsoluble electron acceptor (*11, 12*). In the past, external, soluble redox mediators have consistently been added to MFCs to enhance electron transfer (*4, 13, 14*). A wide number of reports indicates the production of soluble redox mediators by bacteria, which are used to reduce minerals (*15*−*17*). To function, soluble mediators need to meet several requirements (*7*), among which the most important are a sufficient rate of bacterial uptake/excretion and nontoxicity. However, up till now it has not been demonstrated that the presence of soluble, endogenous redox mediators significantly influences electron transfer in MFCs (*18*).

Pseudomonas aeruginosa KRP1 was isolated out of a MFC, in which it was dominantly present (*19*). This species is facultative anaerobic, usually only capable of using oxygen or nitrate/nitrite as electron acceptor (*20*). It is known to produce several phenazine derivatives, the regulation of which is partially determined by quorum sensing (*21*). The phenazine-based pyocyanin is of particular interest for its capability of producing reactive oxygen species (*22*, *23*). Furthermore, this blue component has been shown to exert a significant influence on the respiration of its producer (*15*, *24*). On the basis of these characteristics, it has been postulated that pyocyanin can be used as electron shuttle (*15*). Also several other intermediates in the pathway for pyocyanin and phenazine generation have been reported to hold electrochemical activity and could possibly exert a similar function (*15*). The question therefore rose whether bacteria can produce redox mediators that significantly enhance electron transfer between their cells and an electrode, as present in MFCs. This would enable multiple layers of bacteria to use the electrode in a biofilm. The possibility was recently addressed for insoluble minerals but not demonstrated on significant scale (*15, 16*). To elucidate this, the isolated *P. aeruginosa* KRP1 was used as model organism. The effect of pyocyanin on electron transfer by bacteria was investigated in a heterotrophic microbial fuel cell. In addition, the effect of the compound on the producing species as the interaction with other bacterial species present was assessed.

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TABLE 1. Plasmids and Bacteria Used in This Study with Their Relevant Characteristics

Strains or plasmids	Relevant characteristics	Ref or source
P. aeruginosa		
KRP1	wild type, pyocyanin$^+$, PCN$^+$, isolated out of a microbial fuel cell	19
KRP1-*phzM*	*phzM* mutant of KRP1, obtained by gene replacement using plasmid pZM1-Gm, Gm^R	this study
KRP1-*phzS*	*phzS* mutant of KRP1, obtained by gene replacement using plasmid pZS1-Gm, Gm^R	this study
KRP1-*phzH*	*phzH* mutant of KRP1, obtained by gene replacement using plasmid pMP6010	this study
KRP1-*phzH*|*phz M*	*phzH*|*phzM* mutant of KRP1, obtained by gene replacement using plasmid pMP6010 and pZM1-Gm, Gm^R	this study
7NSK2	wild type, pyocyanin$^+$, isolated from barley roots	34
PHZ1	*phzM* miniTn*pho*A3 derivative of 7NSK2 that is pyocyanin negative, Gm^R	35
E. coli		
ATCC4157	strain obtained from ATCC culture collection	
A. faecalis		
KRA1	wild type, isolated out of a MFC	19
E. faecium		
KRA3	wild type, isolated out of a MFC	19
Lb. amylovorus		
LM1	strain obtained from LabMET culture collection	
Plasmids		
pBR325	suicide vector in *Pseudomonas*, Cm^R/Cb^R/Tc^R	36
pZM1-Gm	2054-bp PCR-amplified fragment of primer pair 4209A-B (phzM) of *P. aeruginosa* PAO1, inactivated by a site-specific insertion of a 803-bp *Not*I-blunted Gm cassette, cloned in pBR325	35[a]
PZS1-Gm	2248-bp PCR-amplified fragment of primer pair 4217A-B (*phzS*) of *P. aeruginosa* PAO1, inactivated by a site-specific insertion of a 803-bp Not1-blunted Gm cassette, cloned in pBR325	b
pMP6010	PCR-amplified fragment of *phzH* of *P. chlororaphis* PCL1391, cloned in the general purpose cloning vector PIC20R	26

[a] Key: Km, kanamycin; Gm, gentamicin; Tc: tetracycline; Cm: Chloramphenicol; Cb, Carbenicilin. [b] T. Pattery and P. Cornelis, personal communication.

Materials and Methods

Setup. MFCs were operated as described by Rabaey et al. (*5*, *19*). The anode and cathode compartments were bolted together with the proton-exchange membrane (Ultrex, Membranes International Inc., Glen Rock, USA) in between. The anode and cathode compartments had a total volume of 30 and 45 mL respectively. In the cathode compartment, aeration was provided at a minimum dissolved oxygen level of 6 mg/L. External electrical contact was provided through the insertion of a graphite rod into the anode, which also functioned as an electrode in the majority of the tests (A = 10 cm^2). For the comparative tests between mixed cultures and *P. aeruginosa* KRP1, 30-cm^2 rough graphite plate anodes were used (Morgan, Grimbergen, Belgium).

The proton exchange membrane was incubated in 2% NaCl solution for 3 h prior to use. For the cathode, a 100 mM phosphate buffer was prepared and enriched with 100 mM of potassium hexacyanoferrate to optimize electrode−oxygen charge transfer (*25*). The setups were installed on a shaker (100 rpm) at 21 °C.

Bacteria. Bacteria and plasmids used are listed in Table 1. Mutant strains *P. aeruginosa* KRP1-*phzM* and KRP1-*phzS* were constructed by homologous recombination using plasmids pZM1-Gm and pZS1-Gm as suicide vector. Mutant strain *P. aeruginosa* KRP1-*phzH* was constructed using plasmid pMP6010 as described by Chin-A-Woeng et al. (*26*).

Production and Purification of Pyocyanin. Pyocyanin was extracted according to Chang et al. (*27*). Briefly, *P. aeruginosa* KRP1 was plated on Pseudomonas agar (Sigma, Bornem, Belgium). After 2 days of growth, the pyocyanin-containing agar was submerged in chloroform, which was subsequently vaporized to concentrate the pyocyanin. The pyocyanin was extracted using an aqueous 0.1 M HCl solution twice, both extracts were pooled. The pH was neutralized using 1 N NaOH, after which a new extraction (twice) with chloroform was performed. The pyocyanin was again concentrated through evaporation, after which petroleum ether was added, and the pyocyanin precipitated using vacuum evaporation. Purity of the obtained pyocyanin was verified using high-performance liquid chromatography (HPLC) (*28*). The column used was a Genesis C18 column (4 μm, 4.6 × 150 mm) (Jones Chromatography, Schoonebeek, The Netherlands) with a UV−vis detector UVD340S (Dionex, Wommelgem, Belgium); for rapid detection, thin-layer chromatography (TLC) analysis was performed according to Fernandez et al. (*28*) with toluene/acetone 3/1 as eluents.

Biofuel Cell Operation. The MFCs were sterilized with ethanol before use. MFCs were operated with several biofuel cell isolates and mutant strains following a standardized procedure. The cultures were grown in nutrient broth (Oxoid, Basingstoke, UK) for 24 h previous to inoculation in the anode compartments (25 mL inoculum). After another 24 h of stabilization, the first feeding (5 mL of glucose solution at a concentration of 2.5 g L^{-1}) was administered after 5 mL sampling. This procedure was repeated daily for a variable number of days, at a loading rate of 0.5 g glucose L^{-1} day^{-1}. Experiments performed using this procedure were (i) testing of the influence of pyocyanin on power output of *P. aeruginosa* KRP1, *A. faecalis* KRA1, *E. faecium* KRA3, *E. coli* ATCC4157, and *L. amylovorus* LM1 and (ii) testing of the mutant strains KRP1-*phzH*, KRP1-*phzM*, KRP1-*phzH*|*phzM*, and KRP1-*phzS*.

For the comparative test between *P. aeruginosa* KRP1, 7NSK2, and PHZ1, glucose was administered daily (loading 1 g glucose L^{-1} day^{-1}) during a 10-day period in MFCs with plate-shaped anodes. Every experiment was performed in triplicate or quadruplicate, controls were grown in serum flasks. Bacterial concentrations were determined during the tests using spectrophotometry at 580 nm (Uvikon, Bunnik, The Netherlands) and verified using plate counts on nutrient agar (Oxoid, Basingstoke, UK).

Electrochemical Monitoring. Measurements of the power output were performed using an Agilent HP 34970 data acquisition unit. Every 30 s, a scan was performed and the data stored. External system resistance R was maximum 100 Ω, current was calculated as

$$I = VR^{-1} = Qt^{-1} \quad (1)$$

with I the current (A), V the voltage (V), Q the charge (Coulomb), and t time (s). Power output of the cells was calculated as

$$P \text{ (watt)} = IV \quad (2)$$

Energy production can then be expressed as

$$E \text{ (Joule)} = Pt \quad (3)$$

Calculations. The amount of pyocyanin added to the reactors can be expressed as a charge quantity (2 mol electrons per mol pyocyanin (*15*))

$$Q = cv2F \quad (4)$$

with c concentration (M), v volume (liter), and F Faraday's constant (96485 C mol^{-1}). This can be compared with the charge as calculated with eq 1. The amount of charge exchanged can be calculated (over chemical oxygen demand COD) to glucose quantity by

$$I = \left(\frac{P}{R}\right)^{1/2} (\text{eq } 1) \rightarrow Q = It \rightarrow m_{\text{glucose}} \text{ (g)} = \frac{Q}{(nf)v} M$$

with n mol electrons exchanged per mol oxygen (*4*), f calculation factor g COD per g glucose (1.06), and M the molar mass of oxygen (32 g/mol).

Microscopy and Flow Cytometry Analysis. Light microscopy was performed using a Polyvar microscope (Leitz, Wetzlar, Germany) with 1000X magnification to verify bacterial growth onto the electrode surfaces and bacterial morphology. Images were taken digitally using a Orca IIIm camera (Hamamatsu, Louvain-La-Neuve, Belgium) branched to a PC. Digital image analysis was performed in MicroImage 4.0 and Microsoft Excel XP.

To analyze the effect of pyocyanin on the different cells, Live Dead staining (BacLight, Molecular Probes, Leiden, The Netherlands) was performed, according to Virta et al. (*29*). In brief, the samples were diluted 100 times, stained with the Live/Dead staining kit and analyzed with a CyanTM LX flow cytometer (Dakocytomation, Heverlee, Belgium) equipped with a 50-mW Sapphire solid-state diode laser (488 nm) by using Summit 3.3 software. Green (FL1) and red (FL3) fluorescence emissions were collected with photomultiplier tubes by using, respectively, 530/40 and 613/20 band-pass filters. Forward-angle light scatter (FSC) was collected by using a type BP488/10 band-pass filter. The FSC neutral-density filter was removed before the bacteria were analyzed. Stabilized isotonic saline was used as the sheath fluid, and a sterile 0.2-μm pore-size filter was piggybacked onto the sheath line. The threshold trigger was set to FL2 (575/25 band-pass filters). The instrument voltage values for FSC, side-angle light scatter (SSC), FL1, and FL3 were optimized for each type of cells.

Cyclic Voltammetry. Cyclic voltammetry was generally performed starting from −450 up to +900 mV and back (*19*). If components are oxidized/reduced during this potential sweep over the culture, current peaks will appear on the voltammogram. Every component that can be reversibly oxidized/reduced has a peak on both the upper and lower curve. If one of the peaks disappears, the component can be regarded as permanently oxidized/reduced. When this technique is performed on a bacterial suspension, peaks can appear for both cellular components such as cytochromes in the periplasm or excreted redox mediators such as pyocyanin. The position of the intersection with the x axis of the connecting line between the upper and the lower peak indicates the formal potential of the component; the size indicates the quantity (*30*). The cyclic voltammetry was performed as described by Park et al. (*31*). For this a potentiostat (model 263a, Princeton Applied Research, Zele, Belgium) branched to a PC was used (Princeton Applied Research, SoftCorr III) at a scan rate of 25 mV s^{-1} in the potential range of −450 to 900 mV. The working electrode was a 5-cm^2 graphite rod, cleaned in ethanol and deionized water prior to use; the counter electrode was a platinum wire, and an Ag/AgCl electrode (MF-2052, BAS, Warwickshire, UK) was used as reference. All three were inserted in the test vial avoiding any contact between the electrodes.

Results

Effect of Pyocyanin Addition on Power Output of *Pseudomonas aeruginosa* KRP1. Sterile MFCs were constructed with rod-shaped anodes. The emphasis was on the sensitive and reliable measurement of electricity production rather than on energy output relative to other systems (*5, 19*). The power output of a MFC-containing *P. aeruginosa* strain KRP1, isolated from the anode compartment of a MFC, was monitored during repetitive feeding cycles and was found to be stable over the testing period in the chosen experimental configuration (Figure 1). The peak power output, as, e.g., seen in Figure 1 from 120 to 130 h, corresponded to 39 μW m^{-2}.

In a follow up experiment, the influence of the mediator level on the electron transfer was studied (Figure 2). After 110 h of normal operation, purified pyocyanin was added to three of the biofuel cells to a concentration of 50 μM. This addition caused an increase in average power output from 116 ± 7 to 672 ± 38 μW m^{-2} (Table 2), yielding a significant increase in cumulative energy output (Figure 2), with an increase of the peak power output from 1206 ± 437 to 2672 ± 889 μW m^{-2}.

Pyocyanin Production by *P. aeruginosa* KRP1. When *P. aeruginosa* KRP1 was grown aerobically (*32*), the production of pyocyanin, phenazine-1-carboxamide (PCN), and phenazine-1-carboxylic acid (PCA) was observed, as verified by HPLC analysis and TLC (data not shown). PCA is the general precursor for the other phenazines mentioned. Out of PCA, 5-MCA is formed (*phzM* gene regulated), which is subsequently the precursor for pyocyanin (*phzS* gene regulated). Alternatively, PCN can be formed out of PCA (*phzH* gene regulated) (*23*).

P. aeruginosa KRP1 was grown in MFCs with and without addition of pyocyanin to the medium. This addition was performed since the regulation of pyocyanin production has been described as self-inductive regulated (*21*). Serum flasks, operated in a similar way as the fuel cell microbial compartments, were used as control reactors. This enabled to discern both the influence of pyocyanin addition and the influence of the growth conditions. Only pyocyanin production was observed in significant amounts in the aqueous phase in the MFCs. No significant phenazine production occurred in the serum flasks (data not shown). The concentrations of pyocyanin measured in solution tended to increase over time for the MFCs (Figure 3). No significant differences in pyocyanin concentration in the aqueous phase were found between the MFCs with or without pyocyanin added (t test, 95% confidence interval). The recovery of the added pyocyanin was generally limited to a maximum of 50%, as evidenced by the data in Figure 3. When the MFC samples were filter sterilized during preparation for HPLC, a blue filter cake was obtained. When samples were centrifuged,

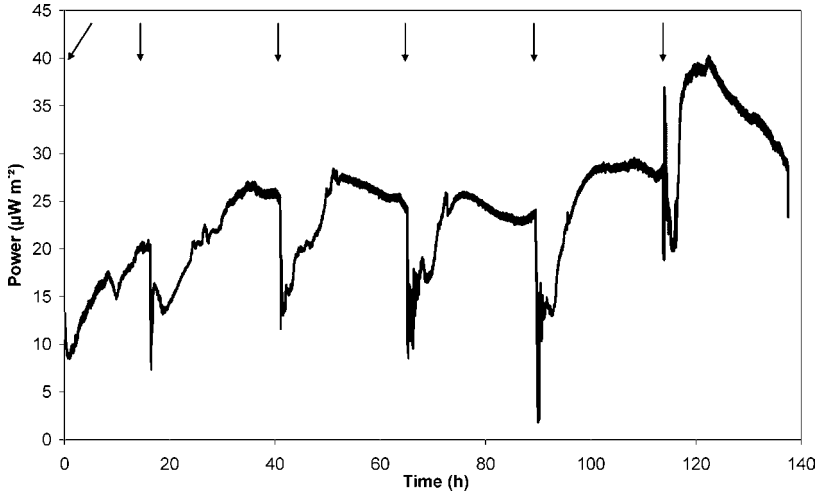

FIGURE 1. Power output (μW m^{-2}) of a glucose-fed (0.5 g glucose L^{-1} day^{-1}) MFC containing *P. aeruginosa* KRP1. Arrows indicate the subsequent glucose spikings.

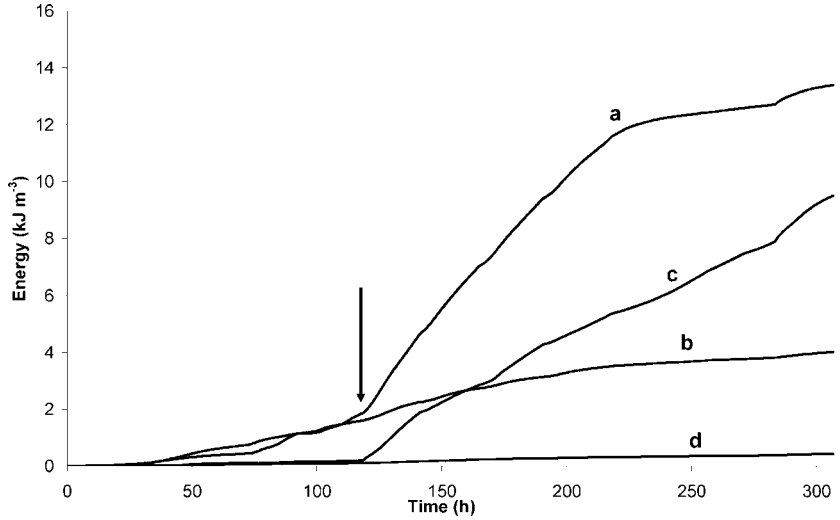

FIGURE 2. Cumulative energy output (kJ m^{-3}) of *P. aeruginosa* KRP1 (a and b) and *Enterococcus faecium* KRA3 (c and d). Reactors a and c received pyocyanin up to a concentration of 50 μM at the time indicated by the arrow.

TABLE 2. Average (Calculated over the Complete Experimental Period) and Peak Power Output (μW m^{-2} of Anode Surface) of MFCs Containing Axenic Bacterial Cultures, with and without Pyocyanin Addition

	average power output (μW m^{-2})		peak power output (μW m^{-2})	
	without pyocyanin	with pyocyanin	without pyocyanin	with pyocyanin
Rod-Shaped Anode				
P. aeruginosa KRP1	116 ± 7	672 ± 38	1206 ± 437	2672 ± 889
KRP1-*phzM*	40 ± 25	95 ± 60	77 ± 10	409 ± 69
KRP1-*phzS*	240 ± 168	27 ± 26	658 ± 158	39 ± 7
KRP1-*phzH*	29 ± 25		1512 ± 35	
KRP1-*phzH*|*phzM*	6 ± 2	57 ± 48	15 ± 1	160 ± 80
E. coli ATCC 4157	117 ± 16	50 ± 53	848 ± 82	236 ± 194
L. amylovorus LM1	86 ± 122	214 ± 180	268 ± 42	1130 ± 73
A. faecalis KRA1	92 ± 34	115 ± 32	442 ± 178	486 ± 103
E. faecium KRA3	12 ± 1	269 ± 19	294 ± 49	3977 ± 612
Plate-Shaped Anode				
P. aeruginosa KRP1	282 ± 27		1670 ± 636	
P. aeruginosa 7NSK2	154 ± 22		330 ± 127	
P. aeruginosa-PHZ1	182 ± 14		258 ± 88	

part of the pyocyanin precipitated with the bacteria. Microscopy examination showed that the bacteria themselves were stained blue. This indicates possible sorption or binding of this component.

The initial bacterial cellular concentration in the MFCs and serum flasks was $8.4 \pm 0.8 \times 10^7$ CFU mL^{-1}. No significant differences in bacterial concentrations after MFC operation were noted with or without pyocyanin addition ($2.1 \pm 1.5 \times$

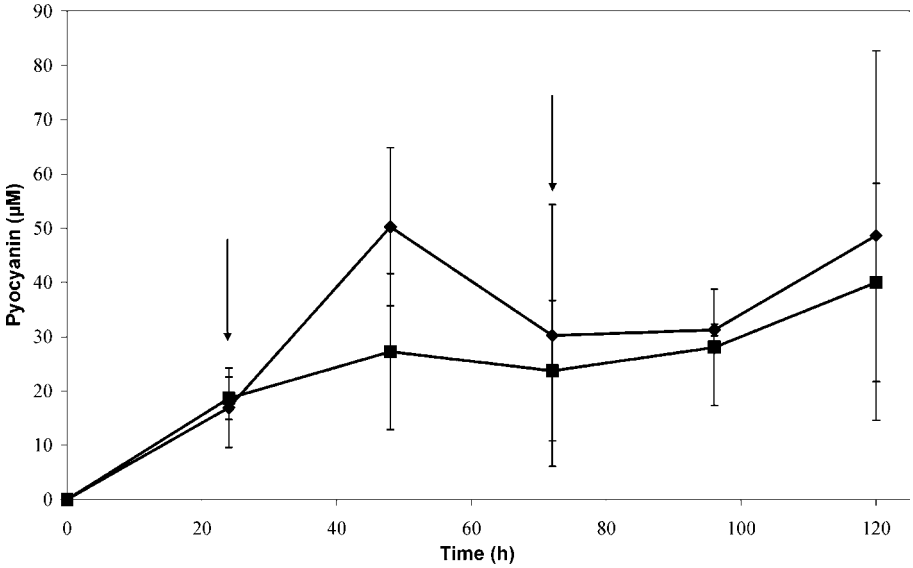

FIGURE 3. Pyocyanin concentrations measured in microbial fuel cells with (◆) and without (■) addition of supplemental pyocyanin (arrows indicate addition of 50 μM pyocyanin).

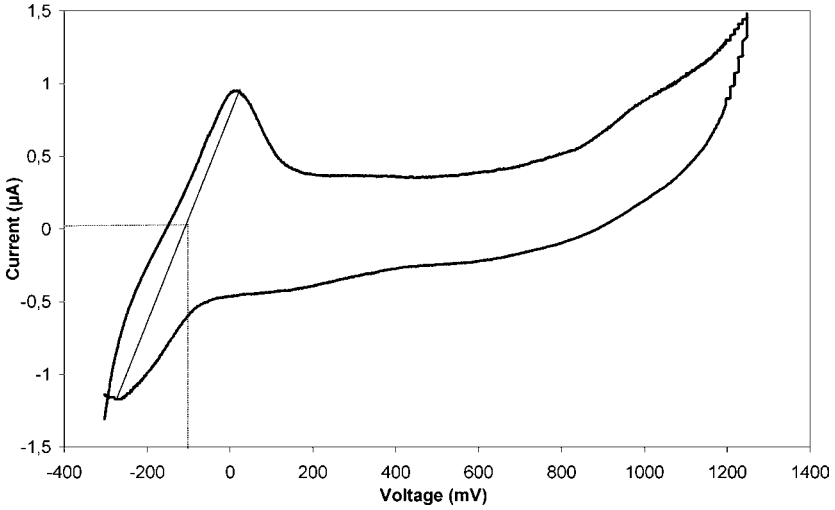

FIGURE 4. Cyclic voltammogram for PCN. The intercept of the line connecting oxidation and reduction peak with the line for current 0 μA indicates the standard potential of the component.

10^8 and $1.6 \pm 0.6 \times 10^8$ CFU mL^{-1} respectively). In comparison, control cultures in serum flasks remained at a bacterial density of $7.79 \pm 1.54 \times 10^7$ CFU mL^{-1} and $5.16 \pm 1.65 \times 10^7$ CFU mL^{-1} with and without pyocyanin addition, respectively.

These findings demonstrate that a biofuel cell, differing from the control reactors only by the presence of an electrode as electron acceptor, stimulates the growth and pyocyanin production of *P. aeruginosa* KRP1. The addition of pyocyanin did not affect final bacterial concentrations.

Power Output of *P. aeruginosa*-KRP1-Based Mutants with or without Pyocyanin. To verify redox activity of the phenazines produced, cyclic voltammetry was performed on phenazine solutions. The analysis showed possible redox mediator activity for pyocyanin, 5-methylphenazine-1-carboxylic acid betaine (5-MCA), and phenazine-1-carboxamide (PCN) (regulated through the genes *phzS*, *phzM*, and *phzH*, respectively) (Figure 4). Mutants were constructed to assess the influence of these phenazines onto the power output (Table 1).

TLC analysis (*28*) showed that mutant KRP1-*phzM* was deficient in the production of pyocyanin and immediate precursor 5-MCA. KRP1-*phzM* showed lower power output than the KRP1 wild type. However, the power output of KRP1-*phzM* was positively stimulated upon addition of pyocyanin to the MFCs. The peak power output increased from 77 ± 10 to 409 ± 69 μW m^{-2}, which resulted in an average power increase over the 145-h experimental period from 40 ± 25 to 95 ± 60 μW m^{-2}. Mutant KRP1-*phzS* was deficient in the production of pyocyanin but still produced 5-MCA as revealed by its red color. KRP1-*phzS* demonstrated, contrarily to the expectations, a decrease in power output upon addition of pyocyanin. The average power outputs were 27 ± 26 μW m^{-2} and 240 ± 168 μW m^{-2} with and without pyocyanin added, respectively.

Mutant KRP1-*phzH* was deficient in the production of PCN but still produced pyocyanin. In MFCs, the growth and power output of KRP1-*phzH* was significantly lower during the initial phase of a test run (29 ± 25 μW m^{-2} in comparison to 114 ± 57 μW m^{-2} for the type strain KRP1). These results indicate a significant influence of the redox-active compound PCN on extracellular electron transfer by *P. aeruginosa*, in addition to pyocyanin.

A double mutant, KRP1-*phzH*|*phzM*, was constructed, which was deficient in the production of pyocyanin, PCN,

and 5-MCA to verify the overall importance of the three redox mediators produced. KRP1-*phzH|phzM* reached a peak power output of only 14.6 ± 0.7 μW m^{-2}, which increased to 160 ± 80 μW m^{-2} upon pyocyanin addition. Average power outputs of 6 ± 2 μW m^{-2} (5% of KRP1) and 57 ± 48 μW m^{-2} were obtained for the MFCs without and with pyocyanin addition, respectively (Table 2).

Effect of Pyocyanin on Power Output of Non-Pyocyanin-Producing Species. The power output with addition of pyocyanin was monitored for the biofuel cell isolates *Enterococcus faecium* KRA3 and *Alcaligenes faecalis* KRA1. Furthermore, *Escherichia coli* ATCC4157 and *Lactobacillus amylovorus* LM1 were included in the tests. For *E. faecium* KRA3, the peak power output increased from 294 ± 49 to 3977 ± 612 μW m^{-2}, a 13-fold increase. This resulted in an increase of the average power output over the 310-h experimental period from 12 ± 1 to 269 ± 19 μW m^{-2}, a 23-fold increase (Figure 2).

For the MFCs containing *E. coli* ATCC4157, the power output was decreased by half by the addition of pyocyanin. The power output was, on average over a period of 115 h, 117 ± 16 μW m^{-2} and 50 ± 53 μW m^{-2} for the reactors without and with pyocyanin addition, respectively. Growth of *E. coli* ATCC4157 (initial bacterial cell concentration 5.2 ± 1.2 × 10^6) was stimulated by the MFCs in comparison to the serum flasks (final bacterial concentrations of 2.2 ± 0.4 × 10^8 and 3.2 ± 0.4 × 10^6 CFU mL^{-1}, respectively). Both for the MFCs and for the serum flask controls, the addition of pyocyanin caused a relative increase in bacterial cell concentrations (final concentrations of 9.5 ± 2.2 × 10^8 with and 2.2 ± 0.4 × 10^8 without addition for the MFCs; 3.0 ± 0.1 × 10^9 with and 3.2 ± 0.4 × 10^6 without addition for the serum flasks). For *A. faecalis* KRA1, no effects were observed on either growth or power output.

L. amylovorus LM1 (inoculated at 8.56 ± 0.97 × 10^7 CFU mL^{-1}) reached bacterial cell concentrations of 2.2 ± 0.1 and 1.6 ± 0.1 × 10^8 CFU mL^{-1} for the serum flasks with and without pyocyanin addition, respectively, after 140 h of growth. When incubated in microbial fuel cells, the numbers of *L. amylovorus* LM1 increased to 2.76 ± 0.04 and 3.18 ± 0.29 × 10^8 CFU ml^{-1} for the MFCs with and without pyocyanin addition, respectively. Clearly, the MFCs allowed increased growth of *L. amylovorus* LM1. Furthermore, addition of pyocyanin to the microbial fuel cells increased the average power output over the 145-h experimental period from 86 ± 122 to 214 ± 180 μW m^{-2} (peak power of 268 ± 42 and 1130 ± 73 μW m^{-2}, respectively). The results are summarized in Table 2.

To corroborate the bacterial counts, the viability of the bacteria was assessed in serum flasks with and without pyocyanin addition. For this, a viability staining was performed and analyzed using flow cytometry. A significant increase in the fraction of live bacteria in pyocyanin-supplemented serum flasks was noted for *L. amylovorus* LM1 (from 46 ± 8 to 79 ± 10%). No significant changes in viability were noted for *E. coli* ATCC4157, *P. aeruginosa* KRP1, and *E. faecium* KRA1. A clear viability decrease was noted for *A. faecalis* KRA1 (from 66 ± 17 to 38 ± 8%).

Power Output of the Isolated *Pseudomonas* Strain vs Other *Pseudomonas* Strains. *P. aeruginosa* strain KRP1 was compared in a biofuel cell with a type strain (*P. aeruginosa* 7NSK2) and with a 7NSK2 deletion mutant, deficient in pyocyanin production (*P. aeruginosa* PHZ1) during a test run of 7 days. Plate-shaped anodes were used for this test to obtain higher output (*5*). The power output of KRP1 was significantly higher than that of the two other strains, on average 282 ± 27 vs 154 ± 22 and 182 ± 14 μW m^{-2} of anode surface, respectively (peak power 1670 ± 636 vs 330 ± 127 and 258 ± 88 μW m^{-2}, respectively). The bacterial density increased from initially 1.2 ± 0.2 × 10^8 CFU mL^{-1} (equal for all strains) to 1.8 ± 0.1 × 10^9 for KRP1, 4.4 ± 0.1 × 10^8 for *P. aeruginosa* 7NSK2, and 6.0 ± 0.1 × 10^8 for *P. aeruginosa* PHZ1. Hence, the anode-isolated strain *P. aeruginosa* KRP1 was able to grow better compared to strain 7NSK2 and PHZ1.

Discussion

This study showed that increased microbial current densities can be obtained due to bacterial redox mediator production in MFCs. The extent of the mediator use by other bacterial species has previously not been described. These findings can explain the higher than anticipated power outputs previously observed for MFCs containing bacterial consortia.

Growth of *Pseudomonas aeruginosa* in MFCs. We recently reported the dominant presence of *P. aeruginosa* KRP1 in an anode-adapted microbial consortium (*19*). Since this organism normally requires oxygen or nitrate/nitrite as an electron acceptor (*20*), this finding opens new perspectives. In a fuel cell, the bacterium uses a particular mechanism to deposit its electrons on a high redox electron acceptor. The active growth observed by the *P. aeruginosa* KRP1 strain in MFCs suggests that it can use the anodic surface as electron acceptor (*19*) to a certain extent, as evidenced by the current generation (Table 2). The power output observed for the isolate KRP1 is a factor of 5 higher than the power output of *Pseudomonas* 7NSK2. Most probably, *P. aeruginosa* KRP1 has adapted to the MFC, in this way facilitating the use of the anode as electron acceptor. *Pseudomonas* species are known to produce redox mediators as pyocyanin, and the MFC anodes regularly colored blue. Hence, the occurrence of this previously doubted strategy (*18*) appears possible.

Generally, the power output of the MFCs showed a peak shortly after feeding. A possible explanation can be an improved contact and mixing in the period after the feeding event. The latter indicates the importance of intensive mass transfer between electron donors, bacteria, and the anode. The effect of the pyocyanin during this first period after the feeding substantiates the importance of the compound as redox mediator, even in high mass-transfer situations.

Pyocyanin Production in Biofuel Cells. The results of the comparative tests, in which *P. aeruginosa* KRP1 was fed either glucose or glucose and pyocyanin, indicate that no significant differences exist in bacterial growth and pyocyanin production (as measured in the aqueous phase) whether KRP1 is grown in microbial fuel cells with or without additional redox mediator dosing. Growth was significantly lower or completely inhibited when the *Pseudomonas* strain was incubated in serum flasks (final bacterial concentrations were a factor of 3 lower in the serum flasks compared to the MFCs). The bacterium was unable to grow substantially under those conditions, which is normal since it does not have a suitable electron acceptor available.

A substantial amount of the pyocyanin appeared to be immobilized on the biomass or taken up by the bacteria, indicating cycling of the compound or immobilization onto the bacterial surface for electron transfer. Further research is needed to verify whether the pyocyanin is actively (by uptake and excretion) or passively (onto the membrane) sorbed.

Addition of Pyocyanin to Mutant *P. aeruginosa* Restored Power Output. The mutant strains, except for the KRP1-*phzS* mutant, demonstrated a significant increase in power output upon addition of pyocyanin. KRP1-*phzH|phzM* was unable to reach acceptable power output levels without pyocyanin present. This demonstrates that the investigated phenazines are essential for *P. aeruginosa* to obtain electron transfer at a significant level.

The observed increase of electrical current is not an effect of irreversible, single-chemical pyocyanin oxidation at the anode after addition. Two moles of electrons can be exchanged per mole of pyocyanin (*15*). The charge exchanged

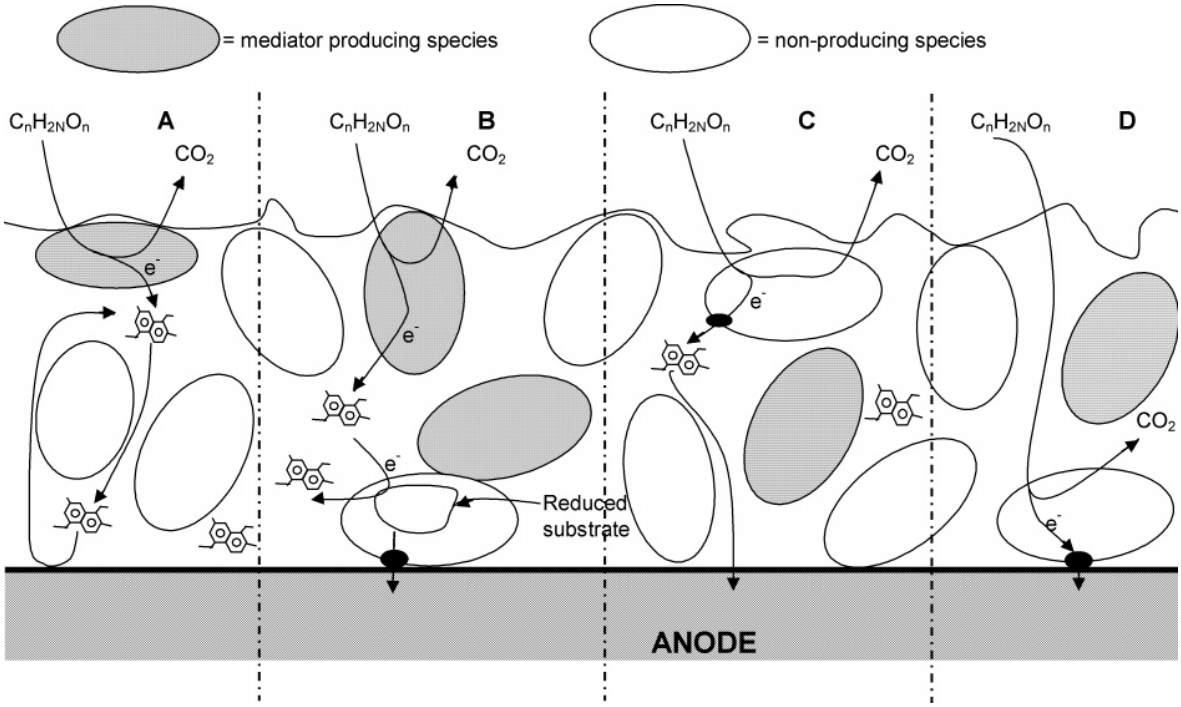

FIGURE 5. Possible working mechanism of self-mediated electron transfer by biofilm bacteria. Grey bacteria are capable of producing soluble mediators; white bacteria are not. Black membrane circles (●) represent membrane-bound compounds capable of transferring electrons from the bacteria to the extracellular environment. (A) Redox mediator producing bacteria use mediators to reach the anode. (B) A bacterium uses a reduced redox mediator as electron donor (hypothesized) and the anode as electron acceptor. (C) A bacterium uses a redox mediator produced by another bacterium to reach the anode. (D) A bacterium transfers electrons to the anode through membrane bound mediators

in the MFC on a daily basis was generally at least an order of magnitude higher than the theoretical pyocyanin charge. For a power output of 100 μW m^{-2}, the pyocyanin shuttle would have been recycled 11 times. This suggests intensive cycling/use of the mediating compound, having a significant influence on the power output. At 3977 μW m^{-2}, the maximal value attained during these experiments, the pyocyanin added (50 μM) would be oxidized/reduced completely in 40 min, enabling a glucose conversion to electricity of 54 mg glucose L^{-1} day^{-1} (CO_2 and H_2O as end products) (for calculations, see materials section), i.e., about 10% of the actual loading rate. Yet, it must be recognized that, in our experiments, only low electron-transfer efficiency, and hence very low energy efficiency, was obtained in comparison to mixed microbial communities and improved reactor designs (*5*, *19*).

The remainder of the glucose conversion can be explained through volatile fatty acids (VFA) formation and aerobic conversion (at a low rate) at the proton exchange membrane, since limited oxygen diffusion through the proton-exchange membrane cannot be totally avoided. Calculations derived from Liu et al. (*6*) (with Nafion proton exchange membrane) indicate possible oxygen influxes of 32−282 mg O_2 L^{-1} d^{-1} for the reactors used in this study. The latter could, in our test runs, relate to 56.4% of the glucose dosed. As the Ultrex membrane is less specific, it can be expected that the oxygen flux is even higher.

Pyocyanin as Electron Mediator for Other Bacteria. The power output and growth of two bacterial strains tested, i.e., *L. amylovorus* LM1 and *E. faecium* KRA3, were stimulated by the addition of pyocyanin to the reactor. This suggests that pyocyanin produced by one bacterium can be also of use for other bacteria present in a mixed consortium. The effect of the mediator appears strain specific, since *E. coli* ATCC4157 showed adverse effects by the pyocyanin addition, while no effects were noted on *A. faecalis* KRA1.

This redox-mediator use can influence the composition of the microbial community by favoring bacteria that use it to reach an insoluble electron acceptor. This correlates with the recent findings concerning mixed microbial community shifts as a result of the addition of pyocyanin (*33*).

Microbial Ecology of Biofuel Cells and Biofilm Reactors. It has been speculated that phenazines could play an important respiratory role in conditions of high bacterial densities, poor mixing, and oxygen limitation (*15*, *16*). Until now, electron transfer in MFCs was considered to occur through direct cell contact (Figure 5D) or by addition of redox mediators. The results from our study demonstrate that (i) bacteria produce redox mediators which have significant effects on the electron-transfer rate (Figure 5A) and that (ii) bacteria can indeed use redox mediators produced by other bacterial species to utilize an insoluble electron acceptor at higher rates (Figure 5C). This enables bacterial species to thrive, or at least survive, in conditions where they normally would have no metabolic advantage. These findings suggest that the influence of soluble electron shuttles within aqueous, subsurface, and microbial fuel cell environments need consideration in terms of current output and processes. Biofilms, growing onto the anode of a MFC, can thus function as a multilayer matrix, in which soluble mediators are embedded. Through these embedded mediators, electrical contact can be present between the top layer of the biofilm and the substrate. This enables the top layer bacteria to use the substrate as electron acceptor and concomitantly have access to the biofilm supernatant electron donors. It is attractive to speculate that other bacterial species down in the biofilm could use the mediators as electron donors and the anodic substratum as electron acceptor (Figure 5B).

This study shows that the power output in MFCs is not solely dependent on a monolayer of bacteria growing onto the anode. Multiple layers of bacteria can possibly participate in the substrate conversion and electron transport toward

the electrode. Hence, the power output attainable can be several times higher than anticipated based on the cellular monolayer configuration.

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