Why are chlorinated pollutants so difficult to degrade aerobically? Redox stress limits 1,3-dichloprop-1-ene metabolism by *Pseudomonas pavonaceae*

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Chlorinated pollutants are hardly biodegradable under oxic conditions, but they can often be metabolized by anaerobic bacteria through organohalide respiration reactions. In an attempt to identify bottlenecks limiting aerobic catabolism of 1,3-dichloroprop-1-ene (1,3-DCP; a widely used organohalide) in *Pseudomonas pavonaceae*, the possible physiological restrictions for this process were surveyed. Flow cytometry and a bioluminescence reporter of metabolic state revealed that cells treated with 1,3-DCP experienced an intense stress that could be traced to the endogenous production of reactive oxygen species (ROS) during the metabolism of the compound. Cells exposed to 1,3-DCP also manifested increased levels of D-glucose-6-P 1-dehydrogenase activity (G6PDH, an enzyme key to the synthesis of reduced NADPH), observed under both glycolytic and gluconeogenic growth regimes. The increase in G6PDH activity, as well as cellular hydroperoxide levels, correlated with the generation of ROS. Additionally, the high G6PDH activity was paralleled by the accumulation of D-glucose-6-P, suggesting a metabolic flux shift that favours the production of NADPH. Thus, G6PDH and its cognate substrate seem to play an important role in *P. pavonaceae* under redox stress caused by 1,3-DCP, probably by increasing the rate of NADPH turnover. The data suggest that oxidative stress associated with the biodegradation of 1,3-DCP reflects a significant barrier for the evolution of aerobic pathways for chlorinated compounds, thereby allowing for the emergence of anaerobic counterparts.

1. Introduction

Chloro-organic compounds rank among the most persistent environmental pollutants [1]. The chemical stability of the C–Cl bond is a major problem, which often causes polychlorinated compounds to be minimally degradable by most environmental micro-organisms [2–4]. Some aerobic bacteria metabolize highly chlorinated organic compounds, including polychlorinated biphenyls [5], haloalkanes [6–8], chlorobenzenes [9,10] and chlorophenols [11,12]. The removal of the halogen substituent occurs through the action of specific dehalogenases [13,14] or as part of a downstream reaction of Cl-containing metabolic intermediates [15]. While Gram-negative aerobic bacteria, in particular pseudomonads, are favourite hosts for designing new biodegradation pathways, attempts to improve the biodegradation of chlorinated compounds through genetic engineering have not had a high degree of success [16,17]. In recent years, however, the number of cases of anaerobic bacteria that can, to some degree, degrade recalcitrant polychlorinated chemicals has increased [18–21]. In these cases, dechlorination of the target molecules occurs through the action of dedicated anoxic dehalogenases [22,23] or as a side effect of organohalide respiration [24], for example, the dehalogenation process in *Dehalococcoides mccartyi* [25,26]. It seems that the natural evolution of aerobic routes for chloro-organic molecules endures more difficulties than the evolution of their anoxic counterparts; however, the precise nature of such bottlenecks has not been clearly documented.
While aerobic metabolism of many organohalides may be biochemically feasible [27,28], their in vivo use is often impossible because of high toxicity. The specifics of the toxicity and the mechanism of the limits on biodegradation in the presence of molecular O₂ are largely unknown. We have examined the physiological regimes experienced by *Pseudomonas pavonaceae* 170 (formerly known as *Pseudomonas cichorii*) when exposed to the highly toxic nematocide 1,3-dichloroprop-1-ene (1,3-DCP). This strain is a Gram-negative soil bacterium that can use some organohalides as a sole carbon and energy source [29]. Because of its carcinogenic properties and widespread occurrence in groundwater supplies, 1,3-DCP is an important environmental pollutant [30]. The key biodegradation steps of this compound involve a hydrolytic dehalogenation reaction executed by the haloalkane dehalogenase DhlA (figure 1). During the process, one of the halogen substituents of the molecule is replaced in a nucleophilic substitution reaction by an H₂O-derived hydroxyl group. Although the biodegradation of 1,3-DCP can proceed to completion, the compound is highly toxic for cells [31,32]. Environmental bacteria thus face the challenge of performing an efficient biodegradation process while enduring the stress associated with the target compound itself and its metabolism.

The bacteria of the genus *Pseudomonas*, as the majority of the heterotrophic bacteria, oxidize organic carbon sources through the Entner–Doudoroff pathway and the tricarboxylic acid cycle [33–35]. D-Glucose is transported through a dedicated ABC uptake system into the cytoplasm, where it is phosphorylated to G6P and D-gluconate-6-phosphate, which can enter into the Entner–Doudoroff pathway and the pentose phosphate pathways. Several of these oxidative steps are coupled to the reduction of NADP⁺ to NADPH, used to generate anabolic precursors needed for bacterial growth and as an antioxidant under stressful conditions (both environmental and endogenous) [36,37]. G6PDH catalyses the NADP⁺-dependent oxidation of G6P and is a key step in the generation of precursor metabolites for the Entner–Doudoroff and the pentose phosphate pathways [38]. The connection between these central catabolic pathways and biodegradation has not been explored in *P. pavonaceae*.

Antioxidant responses in cells exposed to xenobiotics vary widely in the specificity and mechanism of action, but the majority of them rely on the generation of reducing power that the cells use to counteract reactive oxygen species (ROS) production. The appropriate balance of NADPH/NADP⁺ is a central feature to the continued performance of virtually any aerobic micro-organism [39]. A reductive intracellular environment mediated by an appropriate supply of NADPH enables growth and provides the reducing power needed by the cell to counteract the accumulation of ROS generated as a direct consequence of oxidative phosphorylation (a process inherent to the generation of ATP), as well as the ROS derived from the environmental stresses mentioned above [16]. The effectiveness of the vast majority of ROS-detoxifying enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, largely depends on the intracellular availability of NADPH.

The data below document the effects of 1,3-DCP on the cell physiology and oxidative stress responses of *P. pavonaceae*. Specifically, we observed that this micro-organism undergoes a severe metabolic stress when exposed to 1,3-DCP and that its ability to defeat such a condition arises, at least partially, from the modulation of the G6PDH activity. Accumulation of D-glucose-6-P (G6P) as a source of electrons...
was also observed under stressful conditions. These results explore some of the difficulties of degrading chlorinated compounds aerobically and provide a rationale for the emergence of alternative anaerobic pathways.

2. Material and methods

(a) Bacterial strains, culture media and growth conditions

The isolation and some phenotypic properties of the 1,3-DCP degrader P. pavonaceae 170 were described by Verhagen et al. [40]. This strain was used throughout this study. Escherichia coli DH5α (F' proA+ proC+ proU endA1 hsdR17 (rK, mK) recA1 araD-araB leu2-3, -11, -12 thi-1 lacY17, ara-leu8079 galK rpsL15080), P. pavonaceae degrader was used throughout this study. 

Commercial glass vials with teflon-stoppered metallic screw caps (Sigma-Aldrich Co., St Louis, MO, USA) were used for headspace gas chromatography (GC), and we used this caps (Sigma-Aldrich Co., St Louis, MO, USA) were used for headspace gas chromatography (GC), and we used this protocol. Plasmid pSEV A226 contains a luciferase (luxC, luxD and luxE) with the same enzymes. Plasmid pSEV A226E was used as the template in a PCR amplification with corresponding sequences). This DNA fragment was digested with the same enzymes and ligated into pUC18 mini-Tn5 vector (underlined in the corresponding sequences). This DNA fragment was digested with SacI and SpeI and ligated into pUC18-mini-Tn7-Gm-lux [50], restricted with the same enzymes. This intermediate plasmid was used as the template in a PCR amplification with oligonucleotides PDM-F1 (5'-AGT GAG CTC GTT GAC AAT TAA TCA TCG GCA TAG-3'), PDM-R1 (5'-CTG AGT GGT TTA CCT CAC CTC GTC TTA TAC TAC GCC GAT ATA TCA TGC CGA TGA TTA ATT GTC AAC GAG CTC ACT-3') and PDM-R2 (5'-CTG AGT GAG CTC GTT GAC AAT TAA TCA TCG GCA TAG-3'). These procedures resulted in pSEVA226e, in which the expression of luxCDABE is under the control of the constitutive EM7 promoter. This promoter possesses medium strength in Pseudomonas species (D. Pérez-Pantoja et al. 2012, unpublished data). Plasmid pSEVA226e was introduced by electroporation into P. pavonaceae cells rendered electrocompetent by washing the biomass with 0.3 M sucrose, as described by Choi et al. [52].

In the experiments to determine the growth kinetic properties of P. pavonaceae under different culture conditions, the OD600 of each culture condition was determined using a SpectraMax Plus384 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 15 min intervals during 24–36 h. Clear polystyrene 96-well microtitre plates (Nunc A/S, Roskilde, Denmark), containing 200 μl of the corresponding culture medium initially inoculated at an OD600 of approximately 0.05 units, were shaken for 30 s before each measurement; homogeneous conditions were ensured during the cultivation period. Turbidity measurements were computed during exponential growth, and the specific growth rate (μ) was calculated for each condition as μ = ln(OD600 at t1) − ln(OD600 at t2)/(t1 − t2). Normalized growth coefficients were calculated as 100 × (μexperimental/μcontrol), where μexperimental and μcontrol are the specific growth rates under stressful and control (i.e. in the absence of the stressor) growth conditions, respectively. The extension of the lag phase in each case was analytically obtained as detailed by Dalgaard & Koutsoumanis [45].
indicated, 1,3-DCP or diamide was added, and the incubation was continued at 30°C, with shaking at 200 rpm every 15 min. Growth and light emission were recorded in a Varian® 1420 multi-label counter and plate reader (PerkinElmer, Inc., Waltham, MA, USA) and analysed using the WorksOUT applications data analysis software (Wallac Oy, Turku, Finland).

(c) Preparation of cell-free extracts and in vitro determination of enzyme activities and intracellular metabolites concentration

Cell-free extracts were prepared from cells grown to the mid-exponential phase (OD600 of approx. 0.4–0.6 units) and harvested by centrifugation from an appropriate culture volume (usually between 5 and 25 ml) at 4000g at 4°C for 10 min. The pellets were suspended in one volume of 10 mM sodium phosphate buffer (pH 7.5, previously refrigerated) containing 10 mM 2-mercaptoethanol and centrifuged as described earlier. The cells were resuspended in a 0.3–0.5 volume of the same buffer at approximately 0.5 g wet cells ml−1 and sonicated intermittently for 3–5 min with successive sonication (1 min) and cooling (1 min) cycles. A similar procedure was used to prepare cell-free extracts for haloalkane dehalogenase activity determination, but the cells (harvested at different time points) were washed and resuspended in 0.3–0.5 volume of a buffer composed of 10 mM Tris-HCl buffer (pH 8.2), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.02% (w/v) NaN3 and 10% (v/v) glycerol. In both cases, the sonicated cell preparations were centrifuged at 7500g at 4°C for 30 min to remove cell debris. The protein concentration in the cell-free extracts was measured using the Bradford method [53] (with Coomassie brilliant blue G-250), using a kit from Sigma-Aldrich Co. and with crystalline bovine serum albumin as the standard.

The G6PDH activity was determined by following the rate of NADP+ reduction at 340 nm at 30°C in a reaction mixture (1 ml final volume) that contained 50 mM phosphate buffer (pH = 7.5), 10 mM MgSO4, 0.75 mM NADP+, 2 mM G6P and 8–100 μl of the cell-free extract. The rate of the reaction was started by the addition of 0.15 mM NADPH, and an extinction coefficient of 6.22 mM−1 cm−1, representing the difference between the extinction coefficients of NADP+ and NADP++, was used to calculate the specific enzymatic activity. One unit of G6PDH activity was defined as the quantity of enzyme that catalysed the formation of 1 μmol of product for 1 min at 30°C. Haloalkane dehalogenase assays were performed by incubating an appropriate amount of cell extract with 3 ml of a 5 mM substrate (either 1,3-DCP or 1-chlorobutane) solution in 50 mM Tris-HCl buffer (pH = 8.2) at 30°C. Halide liberation was monitored colourimetrically, using a modification of the method described by Bergmann & Sanik [54] as described by Nikel & de Lorenzo [44], and corrected in each experimental condition by the signal obtained using un-inoculated MMY medium. One unit of activity was defined as the quantity of enzyme that catalysed the formation of 1 μmol of halide during 1 min at 30°C. Under the assay conditions described in this study, one unit of G6PDH activity = 1 unit of haloalkane dehalogenase activity = 16.67 nkat.

For the determination of intracellular metabolites, cell lysates were cleared by centrifugation at 13 000g at 4°C for 15 min to remove cell debris and to reduce the NADP+/NADPH background in the extract. The resulting cell-free extracts were boiled for 10 min, chilled in an ice bath and centrifuged at 13 000g at 4°C for 15 min, and the supernatants were immediately used for determination. Appropriate cell-free extract samples (50–350 μl) were incubated at 30°C in a reaction mixture that contained 50 mM Tris HCl (pH = 7.4), 15 mM MgCl2, 0.75 mM NADP+ and 0.5 μM G6PDH from Saccharomyces cerevisiae (Sigma-Aldrich Co.). G6P concentration was spectrophotometrically assessed at 340 nm. α-Gluconate-6-P concentration was determined by applying the procedure as for G6P except that α-gluconate-6-P dehydrogenase (0.025 U ml−1) was used instead of G6PDH. Authentic G6P and α-gluconate-6-P standards (≥99% purity, Sigma-Aldrich Co.) were used at concentrations ranging from 0.005 to 0.25 mM to calibrate the assay, and some experimental samples were spiked with known amounts of metabolites to confirm that boiling did not affect their stability in the heat-treated cell-free extracts. Metabolite concentrations, expressed as nmol mg protein−1, were calculated from the corresponding ΔA450, the extinction coefficients of NADPH and NADH, and the protein concentration in the cell-free extracts.

(d) In vitro determination of the total hydroperoxide content

Hydroperoxides can oxidize Fe2+ to Fe3+ in acidic solution. The latter ions, in the presence of xylene orange [3,3′-bis(N,N-bis(carboxymethyl)aminomethyl)-6-cresolsulfonephthalein disodium salt], form a stable Fe3+-xylene orange complex that absorbs at 560 nm. We used a modification of the procedure described by Jiang et al. [55] to estimate the total hydroperoxide content in the cell-free extracts. The FOXII reagent, which consisted of 0.15 mM xylene orange, 0.25 mM (NH4)2Fe(SO4)2·6H2O, 4 mM 2,6-bis(1,1-dimethylpropyl)-4-methylen and 25 mM H2SO4 (in 90% (v/v) CH3OH), was freshly prepared for each set of determinations and stored at −20°C for no longer than 24 h. For measurements, 75–150 μl of cell-free extract (obtained as described in the preceding section, and containing a fixed amount of soluble proteins) was pipetted into a 1.5 ml microtube and, when necessary, made up to 150 μl with CH3OH, to which 1350 μl of FOXII reagent was added. The reaction mixtures were thoroughly mixed by vortexing and incubated in the dark at room temperature for 45 min. Absorbances were read at 560 nm against a blank of 150 μl CH3OH and 1350 μl FOXII reagent. A calibration curve was run with each set of experimental determination with tert-butyl hydroperoxide (Sigma-Aldrich Co.) as the standard. An extinction coefficient (εFOXII) of 4.52 mM−1 cm−1, representing the intrinsic absorbance of the Fe3+-xylene orange complex, was used to calculate the content of hydroperoxides in the samples, and the results are expressed as molar equivalents of tert-butyl hydroperoxide.

(e) In vivo determination of oxidative stress by flow cytometry

Fluorescence-activated cell sorter cytometry analysis was performed using the ROS-activated green fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Sigma-Aldrich Co.). Pseudomonas pavonaceae was grown in M9 minimal medium containing the corresponding carbon source at 30°C to the mid-exponential phase, when the cell suspension was split into two cultures. One culture was a control, and the other was amended with 0.5 mM 1,3-DCP. Both cultures were incubated, as described earlier, for 1.5 h, and the cells from 0.5–1.5 ml culture aliquots were pelleted by centrifugation at 8000g during 5 min in a benchtop microfuge (Eppendorf centrifuge 5415D; Eppendorf North America, Inc., Hauppauge, NY, USA), washed once with PBS, resuspended in PBS containing 20 μM H2DCF-DA (added from a fresh 4 mM stock solution in dimethyl sulfoxide), and incubated in the dark for 15 min at 30°C. The cell suspensions were diluted 1:500–1:1000 in PBS, as needed, just prior to the measurements. Flow cytometry analysis of the fluorescence levels was performed in a Gallios flow cytometer (Beckman Coulter Inc., Indianapolis, IN, USA), equipped with an argon ion laser of 15 mW at 488 nm as the excitation source. The H2DCF-DA fluorescence emission at 530 nm was detected using a 530/30-nm band pass filter array. Size-related forward scatter signals gathered by the cytometer were used by the Cytlogic v. 1.2.1.
software (CytoFLO Ltd., Turku, Finland) to gate fluorescence data from bacteria in the stream, thus avoiding the mixing of data from the bacteria with data from smaller, non-living particles in the suspension. Data for 25,000 cells per experimental condition were collected, and the CyTOFQC v.1.2.1 software was used to calculate the geometric mean of fluorescence per bacterial cell and the percentage of H$_2$DCF-DA$^-$-cells in each sample.

(f) Analytical procedures

Headspace GC–MS analysis of 1,3-DCP was performed using a Varian3000 gas chromatograph and a 4000MS ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), equipped with a Combi PAL auto sampler (Altmann Analytik GmbH & Co. KG, Munich, Germany). The sample volumes of 100 µL were injected at 250°C (injector temperature) without split. The gas chromatograph was equipped with a 30 m × 0.25 mm i.d. × 0.25 µm FactorFour 5 ms column (low polarity phase, 5% phenyl and 95% dimethylpolysiloxane, Bruker Daltonik GmbH). The interface temperature was adjusted to 230°C, and the oven temperature was set to 200°C. The helium carrier gas was set to a constant flow of 0.6 ml min$^{-1}$. After 6 min of constant heating at 35°C, the oven temperature was raised in a temperature ramp of 10°C min$^{-1}$ to 250°C. To equilibrate the system for the next injection, the temperature was set to 35°C for 10 min. Mass spectra were recorded at two scans per second, with a scanning range of m/z = 50–301. The evaluation of the chromatograms was performed with the 

3. Results

(a) Characterization of growth parameters and haloalkane dehalogenase activity in batch cultures of 

*Pseudomonas pavonaceae* containing $\delta$-glucose as the primary carbon source

*Pseudomonas pavonaceae* 170 was isolated from soil samples heavily contaminated with 1,3-DCP and 1,2-dichloropropane [40]. This Gram-negative micro-organism has been classified as *Pseudomonas cichorii*, using a phenotypic characterization strategy [29], and the genes encoding the haloalkane dehalogenase activity that enables growth on 1,3-DCP were traced to a self-transmissible plasmid that also confers resistance to Hg$^{2+}$ salts [40]. Strain 170 also grows on 3-chloroallyl alcohol, 3-chloroacrylic acid and several $1$-halo-$n$-alkanes [56,57]. The structural and mechanistic characteristics of haloalkane dehalogenase enzymes helped to elucidate the 1,3-DCP biodegradation pathway (figure 1). The first step in 1,3-DCP biodegradation in *P. pavonaceae* 170 is catalysed by a hydrolytic haloalkane dehalogenase (DhaA, E.C. 3.8.1.5) with broad substrate specificity [22]. After two sequential oxidation steps catalysed by an alcohol dehydrogenase and an aldehyde dehydrogenase, the first intermediate metabolite (3-chloro-2-propene-1-ol) is converted into 3-chloro-2-propenal, and finally into 3-chloro-2-propenoic acid. The latter compound, also termed 3-chloroacrylic acid, is the substrate for a specific 3-chloroacrylic acid dehalogenase (CaaD, E.C. 3.8.1; composed by CaaD1, the $\alpha$ subunit and CaaD2, the $\beta$ subunit), which yields 3-oxopropanoate (malonate semialdehyde). Neither DhaA nor CaaD seem to need cofactors for the dechlorination reactions that they catalyse, and the cofactors for Aldh and Adh have not been identified. In the last step of 1,3-DCP biodegradation and mineralization, 3-oxopropanoate can enter into the central metabolic pathways as acetaldehyde upon decarboxylation by a malonate semialdehyde decarboxylase [58,59].

Little is known about the physiological responses elicited in native organochloride-degrader micro-organisms when exposed to 1,3-DCP. We evaluated the growth, consumption of the primary carbon substrate and organohalide biodegradation in batch cultures of *P. pavonaceae* developed in MMY, a semi-synthetic culture medium containing 30 mM $\delta$-glucose as the primary carbon source. Microbial growth was completely inhibited when the 1,3-DCP concentration was higher than 0.5 mM, regardless of the primary carbon source used (data not shown), and we routinely used this concentration in the experiments involving addition of 1,3-DCP. It was proposed that growth inhibition is primarily caused by the toxic effect of 1,3-DCP itself, rather than by the intermediate metabolites derived, because the corresponding 3-chloroallyl alcohols could serve as growth substrates at concentrations up to 5 mM [57]. In cultures containing $\delta$-glucose as the primary carbon source, cells started to grow after a lag phase of 2.8 ± 0.4 h with $\mu = 0.27 ± 0.04$ h$^{-1}$ and reached the stationary phase after approximately 20 h (figure 2a). The OD$_{600}$ of these cultures increased to 1.38 ± 0.16 units between 20 and 48 h (an OD$_{600}$ value approx. 1.6-fold higher than that at 24 h), but at a much slower rate. The kinetics of 1,3-DCP biodegradation followed a distinguishable behaviour throughout the cultivation period. We detected a lag phase during which the organohalide substrate was not consumed. The residual 1,3-DCP concentration did not noticeably change over the first 6 h (probably as a consequence of the low cell density within this timeframe) and started to trend sharply downwards thereafter (figure 2a). The highest rate of 1,3-DCP degradation was registered between 12 and 48 h, and at the end of this cultivation period, the residual organohalide concentration reached 0.154 ± 0.042 mM, indicating that more than 72% per unit of the initial 1,3-DCP was consumed with a specific consumption rate of 85 ± 3 µmol OD$_{600}$ h$^{-1}$. During exponential growth, the specific rate of $\delta$-glucose consumption was 6.8 ± 0.4 mmol OD$_{600}$ h$^{-1}$, and after 18–20 h, most of the primary carbon source was consumed (>90%).
data not shown). By contrast, the specific rate of D-glucose consumption in control cultures, in which no 1,3-DCP had been added, reached 5.1 ± 0.2 mmol OD₆₀₀ h⁻¹. These results suggest that 1,3-DCP can be used with D-glucose as a carbon source and that the presence of an organochloride in the culture medium stimulates D-glucose utilization.

To test whether the uncoupling between growth and 1,3-DCP consumption in P. pavonaceae may arise from a carbon catabolite repression phenomenon mediated by D-glucose, we measured the specific haloalkane dehalogenase activity during the cultivation period (figure 2b). This type of global regulation is typical in biodegradation pathways of environmental bacteria, and the expression of the pWW0-encoded TOL functions for aromatic catabolism in P. putida mt-2 is a paradigmatic example [60]. We observed that the enzymatic activity did not vary significantly between 8 and 48 h (p > 0.1), peaking at (469 ± 75) × 10⁻³ units mg⁻¹ protein⁻¹ at the end of the cultivation period. When the specific haloalkane dehalogenase activity was quantified in cell-free extracts obtained from exponential cultures developed on D-glucose or citrate (i.e. no 1,3-DCP added), values similar to those in cultures amended with the organohalide were obtained ((279 ± 12) and (381 ± 43) × 10⁻³ units mg⁻¹ protein⁻¹, respectively). These experimental values are within the range reported in the literature for cultures of P. pavonaceae, developed on several organohalide compounds as the carbon and energy sources [29,57], and they indicate that haloalkane dehalogenase activity is constitutively present in this micro-organism under both glycolytic and gluconeogenic metabolic regimes.

(b) Addition of 1,3-dichloroprop-1-ene to Pseudomonas pavonaceae cultures has a different effect on growth parameters, depending on the primary carbon source used for growth

We investigated the consistent lag phase in growth and 1,3-DCP consumption, which occurs even when the haloalkane dehalogenase activity is constitutively present in P. pavonaceae. The cells were grown batchwise on two glycolytic substrates (i.e. D-glucose or D-gluconate) or two gluconeogenic substrates (i.e. citrate or succinate) as the primary carbon sources, and the extent of the lag phase and the normalized growth coefficient were determined in each condition (figure 3). Glycolytic carbon sources supported microbial growth, at specific growth rates very close to those observed in the absence of 1,3-DCP, with normalized growth coefficients of 92 ± 3%...
and 85 ± 5% for D-glucose and D-gluconate, respectively. The lag phase before exponential growth of the cells was less than 4 h for both carbon sources. By contrast, cells grown on citrate or succinate exhibited a lag phase 2.5- and 2.1-fold longer, respectively, than that observed on D-glucose (p < 0.05). Judging by the growth kinetics in these batch cultures, cells grown on gluconeogenic carbon sources were also more affected by the presence of 1,3-DCP than those grown under a glycolytic regime, resulting in normalized growth coefficients of 62 ± 3% and 71 ± 4% for citrate and succinate, respectively (p < 0.01, when compared with the normalized growth coefficient observed for D-glucose cultures). As an additional control of the differences on the kinetic parameters caused by using different carbon sources, we compared the normalized growth coefficients for D-gluconate, citrate and succinate, when compared with D-glucose cultures. The values obtained ranged between 85 per cent and 108 per cent, suggesting that the reduction in the specific growth rate in the presence of 1,3-DCP can be predominantly attributed to the organochloride presence in the culture medium.

(c) 1,3-Dichloroprop-1-ene elicits stressful conditions in *Pseudomonas pavonaceae* under both glycolytic and gluconeogenic regimes

In addition to the metabolic state of the cells, we considered the possibility that the addition of 1,3-DCP might impose stressful conditions on *P. pavonaceae*. To test this scenario, we quantified the percentage of cells that gave a positive signal for H$_2$DCF-DA (a molecular probe highly sensitive to intracellular ROS) using flow cytometry, as an estimation of the stress level (figure 4a) upon exposure of the cells to the organochloride. The total percentage of H$_2$DCF-DA$^+$ cells increased under glycolytic and gluconeogenic conditions when 1,3-DCP was added to the cultures. The increase was significantly greater in cells growing on citrate as the primary carbon source (a 6.1-fold increase in the median value of H$_2$DCF-DA$^+$ cells), compared with that in cells growing on D-glucose (a 4.2-fold increase in the median value of H$_2$DCF-DA$^+$ cells). A stressed subpopulation of cells, representing a median value of 59 per cent of the cell count, was detected after the addition of 1,3-DCP under gluconeogenic growth conditions, as opposed to 32 per cent under glycolytic conditions.

The next question was whether this stressed state translates into damage to the cells when exposed to 1,3-DCP. The level of oxidation of unsaturated lipids, which is a process that involves an allylic hydrogen atom abstraction, an insertion of molecular O$_2$, and a subsequent reduction of the resulting hydroperoxyl radical to a free hydroperoxide, is widely used as an estimation of the cellular damage imposed by compounds that elicit oxidative conditions in *vivo* [61,62]. Considering the differences in the content of ROS determined at the single-cell level, we quantified spectrophotometrically the content of hydroperoxides in cultures of *P. pavonaceae*, growing under glycolytic and gluconeogenic conditions, as a consequence of 1,3-DCP addition (figure 4b). Consistent with the changes in stressed populations, as analysed by flow cytometry, we observed a significant increase in the hydroperoxide content in cells exposed to 1,3-DCP, compared with that in untreated cultures. While the hydroperoxide content increased 10.4-fold in D-glucose-grown cells upon addition of 1,3-DCP, the difference between 1,3-DCP-amended and untreated cultures developed on citrate reached 17.6-fold. These results show that cells growing under the metabolic regimes tested have different abilities to use 1,3-DCP as a secondary substrate and to withstand the stressful conditions imposed by the organochloride substrate.

(d) Bacterial luciferase activity correlates with an altered redox state upon exposure of the cells to 1,3-dichloroprop-1-ene

Light emission by bacterial luciferase has been used in a variety of applications, including biosensors and reporters for gene...
expression, which resulted in useful whole cell-based assays for high-throughput screening and environmental monitoring [47,63]. Galluzzi & Karp [64] observed that light emission is strongly dependent on the redox state of cells (specifically to NADPH availability), a characteristic that to a degree limited the wide application of lux-based reporters. FMNH₂ should continually be supplied in the bacterial cell for bioluminescence to remain at a constant level [65]. Both ATP and NADPH are directly involved in the formation of the luciferase substrates, although they do not directly participate in the light-emitting bioreaction. Therefore, bioluminescence is not an isolated process inside the cells because it is connected in the central biochemical network at the redox and energy homeostasis levels. It is also directly linked with other important pathways, including the tricarboxylic acid cycle, the electron transport chain and the synthesis of fatty acids and riboflavin. In particular, the relationship between the bioluminescence pathway and NADPH availability occurs at different points (figure 5b). NADPH is required to reduce an acyl group, previously transferred from the synthetase to the reductase component for the formation of the aldehyde that serves as the luciferase substrate. Reducing power is also required to regenerate FMNH₂ from its oxidized counterpart (FMN). In most Gram-negative bacteria, this reductive pathway is based on the activity of a flavin reductase enzyme, which uses NADPH as the cofactor [66].

We used the sensitivity of the lux-dependent bioluminescence to monitor the redox state of the cells exposed to 1,3-DCP. *Pseudomonas pavonaceae* was transformed with a reporter plasmid in which the expression of luxCDABE is driven by the EM7 promoter, a synthetic derivative of the EM7 promoter that has constitutive and moderate activity in *Pseudomonas* species. When cells were grown on D-glucose alone, the bioluminescence steadily increased as growth progressed and decreased as the cells transitioned into the stationary phase, as expected for the physiological conditions under which NADPH (as well as O₂ and ATP generation) becomes limiting. By contrast, when 1,3-DCP was added to cells growing exponentially, the bioluminescence dropped to approximately 25 per cent of the initial value almost immediately and remained at very low levels. In a separate experiment, we evaluated the effect of diamide addition on bioluminescence generation. Diamide is a strong thiol-oxidant agent known to deplete NADPH and interfere with the balance between reduced and oxidized glutathione *in vivo* [67]. As expected for an oxidative stress-inducing agent, we observed a strong reduction in bioluminescence levels with the addition of diamide, and the bioluminescence values reached values near zero, which were more apparent than those observed for the 1,3-DCP addition. The bioluminescence began to increase approximately 1 h after the diamide addition, suggesting that cells have a detoxification mechanism(s) that allows them to respond to the oxidative stress caused by the toxicant. These results strongly suggest that 1,3-DCP alters the redox homeostasis in *P. pavonaceae*.

**e** D-Glucose-6-P dehydrogenase is a key metabolic step that enables *Pseudomonas pavonaceae* cells to withstand stressful conditions imposed by 1,3-dichloroprop-1-ene addition

G6PDH is a major dehydrogenase involved in NADPH generation in the biochemical network, and we evaluated its activity in *P. pavonaceae* under different growth conditions to investigate the relationship between enzymatic activity and the stressful conditions elicited by 1,3-DCP. As the complete genome sequence of *P. pavonaceae* is not available, we first explored the presence of a zwf gene (encoding G6PDH) in this micro-organism. The amplification of a 460-bp product by PCR, using primers based on published sequences (details not shown), indicated that *P. pavonaceae* has a zwf gene which exhibits a 93 per cent identity to that of *P. fluorescens* PFO-1 (PFO1_2587) [68], its closer orthologue. When the G6PDH activity was evaluated in cell-free extracts (figure 6a), approximately half of the activity observed in D-glucose-grown cells was present in cells growing under a gluconeogenic regime on citrate (38.2 ± 2.4 nmol min⁻¹ mg⁻¹ protein⁻¹), suggesting that this metabolic step contributes to replenish the pentose pool (and generates reducing equivalents) when hexoses are not available as growth substrates. When 1,3-DCP was added to these cultures, the specific G6PDH activity increased under the glycolytic and gluconeogenic regimes, but the increase in the cells grown on citrate was higher than that observed on D-glucose (1.9- and 1.2-fold, respectively, p < 0.05). In both cases, the differences in enzymatic activity are expected to be accompanied by higher rates of NADPH turnover than under untreated conditions, which is consistent with the sharper stressful effect exerted by 1,3-DCP on citrate-growing cells than on D-glucose-growing cells. As a further evidence of the role of G6PDH on NADPH generation under stressful conditions, we quantified its activity in cells exposed to 0.15 mM menadione (a naphtoquinone known to mediate the generation of superoxide radical [69]), and obtained a pattern similar to the one observed for the cells treated with 1,3-DCP. Addition of menadione to the cells grown on citrate provoked a 4.7-fold increase in G6PDH, compared with a 1.6-fold increase attained in the cells grown on D-glucose. These results demonstrate that the metabolic state of the cells is determinant in their ability to withstand stressful conditions.

**f** Addition of 1,3-dichloroprop-1-ene stimulates D-glucose-6-P accumulation in *Pseudomonas pavonaceae*

We questioned whether the differences observed in specific G6PDH activity among the conditions assayed result in different contents of G6P. The level of G6P in pseudomonads is thought to be determined by the tight metabolic balance that exists between the catabolic and anabolic reactions in the metabolic pathways, including the Entner–Doudoroff pathway, gluconeogenesis and the pentose phosphate pathway [70]. Depending on the carbon source and cellular needs, G6P can be (i) directly derived from D-glucose, (ii) biosynthesized from precursors in the lower metabolism through gluconeogenesis, (iii) oxidized by glycolysis to generate ATP (unlikely in this case because most *Pseudomonas* species lack the 6-phosphofructokinase activity needed to catalyse this transformation [35]) and/or (iv) oxidized through the pentose phosphate pathway (by means of the sequential activity of G6PDH and D-glucanate-6-P dehydrogenase) to generate NADPH. These biochemical steps can occur either separately or in combination, allowing the cells to satisfy the need of metabolic intermediates and to maintain the redox homeostasis.
We monitored the content of this phosphosugar among different metabolic regimes and culture conditions (figure 6b). Under control conditions, the content of G6P reached 11.9 ± 1.2 nmol mg protein⁻¹ in cells grown on D-glucose. The value for cells grown on citrate was lower, as expected for gluconeogenic conditions. It is interesting to note that *E. coli* BW25113, grown in the same culture medium containing 30 mM D-glucose as carbon source, had a G6P content of 4.2 ± 0.3 nmol mg protein⁻¹, which suggests that G6P might have a distinctive metabolic role in *P. pavonaceae* different than that in Enterobacteria. Upon addition of 1,3-DCP, the content of G6P in *P. pavonaceae* increased significantly under both glycolytic and gluconeogenic regimes, mirroring the behaviour observed for G6PDH activity. The intracellular G6P concentration had a 1.4- and 1.8-fold increase in cells grown on D-glucose and citrate, respectively (p < 0.05). These
Note that the superoxide-generating naphthoquinone menadione was added at 0.15 mM to some cultures to impose oxidative stress conditions. In cells grown on D-glucose, D-gluconate-6-bisphosphatase is expected to account for an elevated G6P content (in particular, that of D-fructose-1,6-bisphosphate) when 1,3-DCP was added as a co-substrate, which is consistent with the notion that more G6P is produced by direct phosphorylation of D-glucose. Although the activity of G6PDH expressed in cells grown on D-glucose citrate (i.e. glycolytic and gluconeogenic metabolic regimes) under control conditions (nil) and in the presence of 0.5 mM 1,3-dichloroprop-1-ene (1,3-DCP) increases (Figure 6). In vitro quantification of the specific (Sp) D-glucose-6-P dehydrogenase activity. Note that the superoxide-generating naphthoquinone menadione was added at 0.15 mM to some cultures to impose oxidative stress conditions. (a) Determination of the intracellular D-glucose-6-P concentration. Appropriate aliquots of clarified cell-free extracts were boiled and centrifuged, and supernatants were spectrophotometrically analysed for D-glucose-6-P as detailed in §2. Each bar represents the mean value of the corresponding parameter ± s.d. of duplicate measurements from at least three independent experiments. In both cases, the asterisk marks denote significant differences in the corresponding parameter at either the 95% level (single asterisk) or the 99% level (double asterisks) when compared with the control culture (without additives), as evaluated with ANOVA.

Figure 6. Evaluation of biochemical and metabolomic parameters in P. pavonaceae cells growing exponentially on MMY medium containing either 30 mM D-glucose or citrate (i.e. glycolytic and gluconeogenic metabolic regimes) under control conditions (nil) and in the presence of 0.5 mM 1,3-dichloroprop-1-ene (1,3-DCP). (a) In vitro quantification of the specific (Sp) D-glucose-6-P dehydrogenase activity. Note that the superoxide-generating naphthoquinone menadione was added at 0.15 mM to some cultures to impose oxidative stress conditions. (b) Determination of the intracellular D-glucose-6-P concentration. Appropriate aliquots of clarified cell-free extracts were boiled and centrifuged, and supernatants were spectrophotometrically analysed for D-glucose-6-P as detailed in §2. Each bar represents the mean value of the corresponding parameter ± s.d. of duplicate measurements from at least three independent experiments. In both cases, the asterisk marks denote significant differences in the corresponding parameter at either the 95% level (single asterisk) or the 99% level (double asterisks) when compared with the control culture (without additives), as evaluated with ANOVA.

4. Discussion

The synthetic halogenated aliphatic 1,3-DCP was introduced as a nematocidal soil fumigant in the 1950s, and since then has been used in large quantities [32,71]. The organohalides are recalcitrant to biodegradation for several reasons: (i) they are highly toxic, (ii) most complex organohalides are unnatural compounds recently introduced into the environment by human activities, and micro-organisms are unlikely to possess complete catabolic pathways for their mineralization, and (iii) if degradation is initiated by a dehalogenation reaction catalysed by an existing dehalogenase or oxidase, the lack of enzymes for the rapid conversion of the resulting halogenated alcohols or aldehydes would lead to the accumulation of toxic and/or highly reactive metabolic intermediates. Besides, the chemical bonds in these compounds are extraordinarily stable, making them very persistent in most environments. The slow biodegradation of 1,3-DCP observed in contaminated soils leads to the theory that some micro-organisms have assembled their catabolic pathways recently, possibly by recruiting (pre-) existing enzymes [22,72,73]. The constitutive expression of the haloalkane dehalogenase activity in P. pavonaceae grown on different carbon sources could indicate that the enzymes involved are part of a recently assembled catabolic pathway, which lacks an active regulatory system to tightly regulate the expression of the cognate genes. Pseudomonas pavonaceae thus affords an exceptional experimental system to examine how still-evolving environmental bacteria manage the interplay between different metabolic and stress programmes because some of the growth substrates used by this micro-organism constitute acute physiological stressors. In general, our data reveal that P. pavonaceae cells undergo stressful conditions when facing (and degrading) 1,3-DCP.

Three major types of response to stressful conditions imposed by xenobiotics have been recognized [16]. Metabolic programmes consist of the tuning (both at the gene transcription and regulation of activity levels) of enzyme sets required for the catabolism or anabolism of nutrients and intermediates; stress-response programmes for adaptation to suboptimal growth conditions; and morphological programmes related to shape, transport and surface chemistry of the bacterial cell. These three programmes are closely connected, and their functioning largely determines the survival of a given population in specific niches or its displacement by a fitter organism when facing adverse conditions.

In this study, we focused on the interplay between metabolic and stress-response programmes elicited by 1,3-DCP in P. pavonaceae. As a result of our findings, we hypothesized that the metabolic state of the cells influences their ability to face (and metabolize) the organochloride as an alternative carbon and energy source. The homeostasis conditions within metabolic networks are orchestrated by fine-tuning mechanisms that prevent drastic responses to nutritional or environmental perturbations. Instead of the typical all-or-none behaviour of
most bacterial transcriptome constituents, biochemical networks are regulated by devices that tune (rather than switch) metabolic regimes, according to the overall physiological conditions. These processes allow the cells to generate the appropriate ratios of biosynthetic precursors for balanced growth [74]. As the haloalkane dehalogenase activity is constitutively present in *P. parvocaceae*, the biochemical network in this micro-organism is expected to respond to the presence of 1,3-DCP by adjusting metabolic fluxes to obtain the relevant metabolites and cofactors needed for biodegradation and growth. Our data suggest that in conditions that foster metabolic stress, under both glycolytic and gluconeogenic regimes, G6P is accumulated at high levels and possibly maintained as a reservoir of reducing power. The G6P amassed under such conditions is most likely used as a substrate of G6PDH to generate NADPH. The strong activity of gluconeogenic pathways, needed to fulfil G6P needs when cells are grown on citrate, supports the theory that fluxes throughout the biochemical network are adapted to meet the demand of metabolic precursors under stressful conditions. In addition to these core bioreactions, the need for a fine-tuned balancing of different redox cofactors (i.e. NADH and NADPH) is particularly accounted for in several bacteria by the presence of transhydrogenases, which are enzymes that catalyse the electron transfer reactions between NADH and NADPH to equilibrate the respective pools thus meeting metabolic demands [38]. Although not investigated in this study, transhydrogenases could contribute to the homeostasis of NADH and NADPH together with the major dehydrogenases of the central catabolic pathways.

A possible generalization of the data presented in this study is that xenobiotic-mediated stress sets a limit to the possibilities of evolving aerobic pathways for the degradation of organochloride compounds. Bacteria must evolve adequate biodegradation pathways for the compound at stake, and they will also encounter the metabolic stress imposed by the potential substrate during anoxic metabolic regime. In general, anaerobic metabolisms are less prone to generate oxidative stress [75], and the chemical and thermodynamic itinerary for assembling new biodegradation routes could be less constrained than in aerobicism. On the other hand, the strong electronegativity of multiple chlorine substrates contributes to make an oxidative attack on organochlorides more unlikely than a reductive attack. These results might explain, at least partially, the observed capacity of anaerobes to perform organohalide respiration for processing compounds that are virtually impossible to metabolize in the presence of oxygen. Finally, the results obtained in this study open new avenues for the rational design of biocatalysts tailored for the efficient degradation of organohalides [44].

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