Microbiological versus Chemical Reductive Sulfidation: An Experimental and Theoretical Study

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Oriane Della-Negra[†], Brieuc Le Cacher de Bonneville[†‡], Sébastien Chaussonnerie[†], Denis Le Paslier[*†], Gilles Frison[*‡ǂ], Pierre-Loïc Saaidi[*†]

[†]Génomique Métabolique, Genoscope, Institut François Jacob, CEA, CNRS, Univ Evry, Université Paris-Saclay, 91057 Evry, France

[‡]Laboratoire de Chimie Moléculaire, Ecole Polytechnique, CNRS, IP Paris, Palaiseau, France

[ǂ]Sorbonne Université, CNRS, Laboratoire de Chimie Théorique, Paris, France

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ABSTRACT: Microbiological reductive sulfidation (RS) has rarely been documented, although it represents an efficient strategy for thiol formation. In this work, we reported on the sulfate-respiring bacterium *Desulfovibrio* sp.86 that has previously demonstrated RS activity toward the pesticide chlordecone. The purpose of the study was to assess its substrate-versatility using a set of 28 carbonyls, to compare with chemical RS and to rationalize the observed trends using a dual experimental and theoretical approach. The chemical RS generally proceeds in two steps (S-O exchange using a sulfur donor like P_4S_{10}, reduction of the thione intermediate). Intriguingly, chlordecone was found to be converted into chlordecthiol following the first step. Hence, we designed a protocol and applied it to the 28 substrates to assess their propensity to be directly converted into thiols with the P_4S_{10} treatment alone. Finally, we performed density functional theory calculations on these carbonyls and their thiocarbonyl derivatives to build a set of structural, electronic and thermodynamic parameters. The results showed that chemical and microbiological RS probably involved two distinct mechanisms. Chemically, we observed that several carbonyls, possessing electron-withdrawing groups and/or aromatic rings, were directly transformed into thiols in the presence of P_4S_{10}. The correlation obtained with the electron affinity of the thiones led us conclude that a probable single-electron reductive transfer occurred during the first step. We also found that *Desulfovibrio* sp.86 transformed a variety of aldehydes and ketones, without ever detecting thiones. No significant correlation was observed with the calculated parameters but a relationship between aldehyde RS biotransformation and bacterial growth was observed. Differences in selectivity with chemical RS open the way for further applications in organic synthesis.

1. INTRODUCTION

Among the different strategies for creating a C-S bond[1–9], reductive sulfidation (also referred as reductive thiolation) is one of the most appropriate. It consists in a two-step process: (i) thionation of carbonyls with the use of sulfur donors (*e.g.* Berzelius reagent (P_4S_{10}), hydrogen sulfide, thiophosphoryl chloride, bis(trimethylsilyl)sulfide, rhodamine or 2,4-bis(p-methoxyphenyl)-1,3-dithiadiphosphetane-2,4-disulfide (Lawesson's reagent))[6,10]; (ii) reduction of the thione into the target thiol using $NaBH_4$ or $LiAlH_4$ for example[11,12]. One-step reductive sulfidation has rarely been observed. A few papers have reported this direct transformation using H_2S. However, in these cases the required conditions, that limit the substrate spectrum, have precluded the widespread use of this strategy[13–15]. More recently we have discovered that the single application of P_4S_{10} on chlordecone congeners resulted in a significant amount of chlordecthiols[16].

In biology, the formation of C-S bonds can be catalyzed by several types of enzymes[17,18]. However, only a few EC numbers refer to enzymatic reductive sulfidation processes: (i) conversion of a cysteine into a 3-oxo-alanine residue in the active site of sulfatases (EC 1.8.98.7 and EC 1.8.3.7), (ii) oxidation of methanethiol into formaldehyde (EC 1.8.3.4). These enzymes, specific to a single metabolic substrate, actually promote the backward direction, *i.e.* the formation of aldehydes. More generally, some papers referenced the enzymatic transformation of carbonyls into thiones. For example, 4-thiouridine was obtained from uridine via cysteine, ATP and several proteins[19]. In rare cases, the thiones formed were spontaneously isomerized into thiol moieties via tautomerization steps, just as in the biosynthesis of 2-thioglucose-6-phosphate[20] or molybdenum cofactor[21]. Interestingly, within the biosynthesis of thienodolin by *Streptomyces* sp. FXJ1.172, a formal reductive sulfidation occurred to yield 6-Cl-thiotryptophan from 6-chloroindole-3-pyruvic acid. The authors postulated the concerted action of a dehydrogenase, an isopeptidase and a protein of unknown function encoded in the biosynthetic gene cluster (ThnD-F). In this mechanism, they excluded the intermediate

formation of a thione function[22]. A similar observation was reported for the biosynthesis of the antibiotic chuangxinmycin from tryptophan, by *Actinoplanes tsinanensis*. In that case, the simultaneous presence of two proteins, Cxm3 (unknown function) and Cxm4 (a sulfur carrier protein) was found essential to induce the conversion of the ketone into the thiol intermediate[23].

Recently, we have reported a new example of a bacterial reductive sulfidation: *Desulfovibrio* sp.86 a sulfate-reducing bacterium mediates the transformation of the insecticide chlordecone and its congeners into chlordecthiols (their thiol derivatives), but this time as end-products[16], contrary to the thiol intermediates described in the biosynthesis of thienodolin and chuangxinmycin[22,23]. We observed that the incubation conditions play a critical role (mandatory sulfate-reducing conditions using sulfate, sulfite, bisulfite or thiosulfate as electron acceptors; confined atmosphere (CA) preventing any gas-exchange). Interestingly, isotope labeling experiments revealed that the inserted sulfur atom originated from the inorganic sulfur source used for bacterial growth.

While chlordecone is generally depicted as a perchlorinated ketone[24], its most stable physical state, neat or in solution, corresponds to a monohydrate form, *i.e.* a gem-diol function in place of the carbonyl moiety[25]. Although generally not thermodynamically favored, this hydration can occur spontaneously with certain short-chain and/or electron-deficient aldehydes/ketones. In the case of chlordecone, the ring-constraints of the bishomocubane cycle and the electron-withdrawing effect of the chlorine atoms are presumably responsible for this unusual reactivity.

The purpose of the present work was to evaluate the versatility of the bacterial reductive sulfidation far beyond the structure of chlordecone and to assess its wider applicability. We built a set of 28 aldehydes and ketones and incubated them in presence of *Desulfovibrio* sp.86 as previously described[16]. We hypothesized that similar structural and/or electronic effects might be involved in the bacterial reductive sulfidation and the one-step chemical reductive sulfidation in the presence of P_4S_{10} observed for chlordecone and its congeners. We thus designed an additional experimental approach to evaluate the propensity of the same set of carbonyl compounds to be directly converted into their thiol derivatives using P_4S_{10} alone. We also performed density functional theory (DFT) calculations in order to build for each substrate a set of structural, electronic and thermodynamic parameters. Lastly, we evaluated, using statistical tools, the presumable correlations between bacterial, chemical and theoretical data.

2. RESULTS AND DISCUSSION

2.1. Selection of the target carbonyl compounds

In order to simplify the monitoring of bacterial and chemical reactions, we targeted volatile substrates easily detected using GC-MS technique (Figure 1). According to the structural and electronic particularities of chlordecone (**12a**), we selected a series of aldehydes and ketones containing electron-withdrawing groups (**1a**-**2a**, **13a**-**15a**) (known to be partially or completely hydrated)[25–29] and several cyclic ketones to explore the possible effect of the ring constraints (**20a**-**28a**). Finally, we added aliphatic, conjugated and aromatic aldehydes and ketones in order to enlarge the structural diversity (**3a**-**11a**, **16a**-**19a**). An additional lactone (**29a**, Figure 1) was also tested.

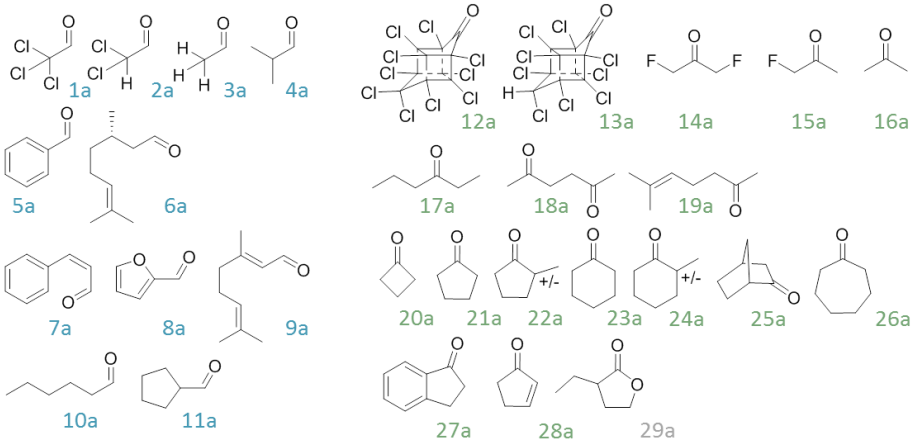


Figure 1. Structures of selected carbonyl compounds. The blue numbers correspond to aldehydes, green to ketones and grey to lactone. For clarity, no hydrated and/or oligomeric forms have been presented.

2.2. Chemical reductive sulfidation of carbonyl compounds using P_4S_{10} and $NaBD_4$

To mimic the bacterial reductive sulfidation observed with *Desulfovibrio* sp.86, a standardized chemical protocol using P_4S_{10} was applied to each selected carbonyl compounds. The chemical reaction conditions were adapted from already published protocols[11,16]. For the first reaction step, P_4S_{10} was used in acetonitrile at room temperature, instead of the most popular condition (refluxing pyridine)[10,30]. Acetonitrile is also reported for this type of reaction[30] and its use at room temperature could prevent chloral or similar

compounds from hydrolysis or dehalogenation, possibly enhanced by high temperatures and the basic conditions[29]. At the end of this step, the thione intermediate was observed in most cases (Figures S6-30). The second step consisted in the reduction of the thiones using deuterated sodium borohydride in anhydrous methanol-d_4. All reactions were monitored using either headspace gas chromatography coupled to mass spectrometry (HS-GC-MS) or GC-MS using liquid injection (see Figures S7-31). In each case, the peak area of the deuterated thiol was compared to the peak area of the non-deuterated thiol so as to assess the proportion of thiol already produced in the first step versus the thiol formed after the addition of the reductant $NaBD_4$ in the second step.

Interestingly, polychloroaldehydes (**1a**, **2a**), chlordecone (**12a**) and 10-monohydrochlordecone (**13a**) were almost completely transformed into their thiol derivatives after the first step. For most of the carbonyl compounds (**3a-5a**, **7a-8a**, **11a, 14a-17a, 19a-27a**) the deuterated thiols proved to be the predominant end-products. The thione intermediates were thus mainly reduced during the second step.

In the case of hexane-2,5-dione (**18a**), citral (**9a**), citronellal (**6a**) and cyclopent-2-en-1-one (**28a**), the expected thiol products were not observed. For each of these four compounds, we proposed a structure based on the analysis of the in-source GC-MS fragmentation and comparison with the existing mass-spectra database and/or literature (Table S6). Treatment of hexane-2,5-dione with P_4S_{10} resulted in the appearance of one new GC-MS peak that remained unchanged after the reduction step. Based on the analysis of its mass spectrum (Figure S13), we assigned it as 2,5-dimethylthiophene[31]. Indeed, it has already been reported that hexane-2,5-dione was transformed into dimethylthiophene (**18b**) by P_4S_{10} via a Paal-Knorr mechanism[32]. For citral, one GC-MS peak was detected after the first step at a retention time of 16.21 min (Figure S22). The associated in-source mass fragmentation matched with p-cymene fragments (**9b,** Figure S22). Just as citral, citronellal was transformed into one major compound (retention time: 16.86 min) that displayed a fragmentation pattern highly similar to that of 3,8-p-menthadiene (Figure S26)[33]. In these cases, the electrophilic phosphorous center of P_4S_{10} probably activates the carbonyl moiety as does the acid catalyst in the known cyclization of citral and citronellal that lead to the same products[34–36]. Cyclopent-2-en-1-one (**28a**) was transformed into a compound that shares the same mass spectrum as 3-mercaptocyclopentan-1-one (Figure S23)[37] (**28b**). A 1,4-nucleophilic addition mechanism would prevail in the expected reductive sulfidation process. Finally, the lactone (**29a**) treated with P_4S_{10} yielded the expected cyclic thionoester (**29b,** Figure S28) as previously observed[38] but no trace of reduced thiol was detected with $NaBD_4$.

2.3. Versatility and specificity of *Desulfovibrio* sp.86 reductive sulfidation toward carbonyls

We modified the incubation conditions previously reported for the reductive sulfidation of chlordecone to test the panel of compounds in the presence of *Desulfovibrio* sp.86[16]. The present protocol consisted in supplying the culture in higher concentrations of sulfate (25 mM) and lactate (50 mM) as electron acceptor and electron donor respectively (see "Experimental section"). The bacterium and the substrate were then incubated in anaerobiosis (N_2/H_2, 98/2), in a 100 mL sealed vial, thus corresponding to a confined atmosphere system (CA). The search and the identification of the expected thiols were facilitated by the thiols obtained chemically using the previous $P_4S_{10}/NaBD_4$ sequence. Every transformation was monitored by GC-MS analysis (Figures S32-52).

Unexpected behaviors were observed for a number of target carbonyl compounds: (i) trichloroacetaldehyde (chloral, **1a)**, dichloroacetaldehyde (**2a**), 1,3-difluoropropan-2-one (**14a**) and 1-fluoropropan-2-one (**15a**) disappeared within a few hours in both biotic experiments and abiotic controls; (ii) acetone (**16a**) and acetaldehyde (**3a**) were no longer detected in the biotic incubations after one week while no thiol derivatives could be observed at the same time. Indeed, it is known that chloral (**1a**) decomposes in water to chloroform and methanoic acid at moderate temperature and under basic conditions[29]. In our case, chloroform was detected in biotic and abiotic experiments that involved chloral (**1a**), confirming the previous report. We thus assumed that chloral (**1a**), dichloroacetaldehyde (**2a**) but also 1,3-difluoropropan-2-one **14a** and 1-fluoropropan-2-one (**15a**) underwent hydrolysis during incubation. Acetone (**16a**) and ethanal (**3a**) are probably used by *Desulfovibrio* sp.86 as carbon sources and/or involved in other metabolic pathways as has already been observed in anaerobic conditions for other sulfate-reducing bacteria[39,40].

After an incubation of two months, no trace of thiol could be detected for the linear ketone (**17a**), the enone (**19a**), the seven-membered ring ketone (**26a)**, the indanone (**27a**) and the lactone (**29a)** while the substrates were still present in the culture. All other cyclic ketones were transformed into their thiol derivatives (**20a-25a**) but incompletely after the same period of time. For cyclopent-2-en-1-one **28a**, the reduction of the C=C double bond was first evidenced with the detection of cyclopentanone (**21a**) which was finally transformed into cyclopentanethiol. The promising high diastereoisomeric excess (70-95%) observed for the reductive sulfidation of nor-camphor (**22a)**, 2-methylcyclopentanone (**24a**) and 2-methylcylohexanone (**25a**) unfortunately did not correspond to a complete conversion. Finally, chlordecone (**12a**), 10-monohydrochlordecone (**13a**) and all aldehydes have completely disappeared to give rise to their thiol derivatives (**4b-13b**). It turned out that citral and citronellal (**9a**, **6a**) gave rise to two GC-MS peaks sharing the same fragment losses with molecular ions m/z 168 and 170 respectively. The mass spectra did not correspond to the expected thiols. However, the analysis of their in-source fragmentation mass spectra showed strong similarities with those from isopiperitenol and isopulegol (Figures S43-44)[33,41]. We thus assumed that a ring-closing reaction occurred leading to cyclic thiols. Finally, dimethylthiophene was detected when hexane-2,5-dione (**18a)** was incubated**.**

2.4. Chemical versus microbiological reductive sulfidation

At that stage, the results of bacterial and chemical reductive sulfidation were compared. First of all, we noticed that four carbonyl compounds possessing electron-withdrawing groups (**1a**, **2a**, **14a**, **15a)** that were not stable in liquid cultures reacted under chemical conditions to give the expected thiols (**1b**, **2b**, **14b**, **15b)** (Figure 2A). So we could not conclude on their fate in the presence of *Desulfovibrio* sp.86. Acetaldehyde (**3a)** and acetone (**16a**) were apparently metabolized by this bacterium but in unidentified products (Figure 2B). A set of five carbonyl substrates (**17a**, **19a**, **26a**, **27a**, **29a)** were chemically transformed but remained unchanged under microbiological conditions (Figure 2C). A total of six aldehydes **4a**, **5a**, **7a**, **8a**, **10a**, **11a** and nine ketones **12a**, **13a**, **18a**, **20a**, **21a**, **22a**, **23a**, **24a**, **25a** reacted similarly both under chemical and bacterial conditions (Figure 2D), whereas the two aldehydes **6a**, **9a** and the enone **28a** did not lead to the same final products depending on the conditions (Figure 2E).

Interestingly, even if microbiological transformations were not total, their diastereomeric excesses were systematically higher than those obtained under chemical conditions (Table 1). Furthermore, when the biotransformation of carbonyl compounds to thiol derivatives was effective, no trace of the possible thione intermediate was detected, contrary to the chemical reductive sulfidation. In addition, all negative controls performed without bacteria never showed any reductive sulfidation activity. It is noteworthy that the microbiological reductive conditions were reached through the use of Na_2S. We thus concluded that this possible sulfur-donor reagent, known to act on several ketones in organic media[12], is not reactive in the present case with all the compounds studied.

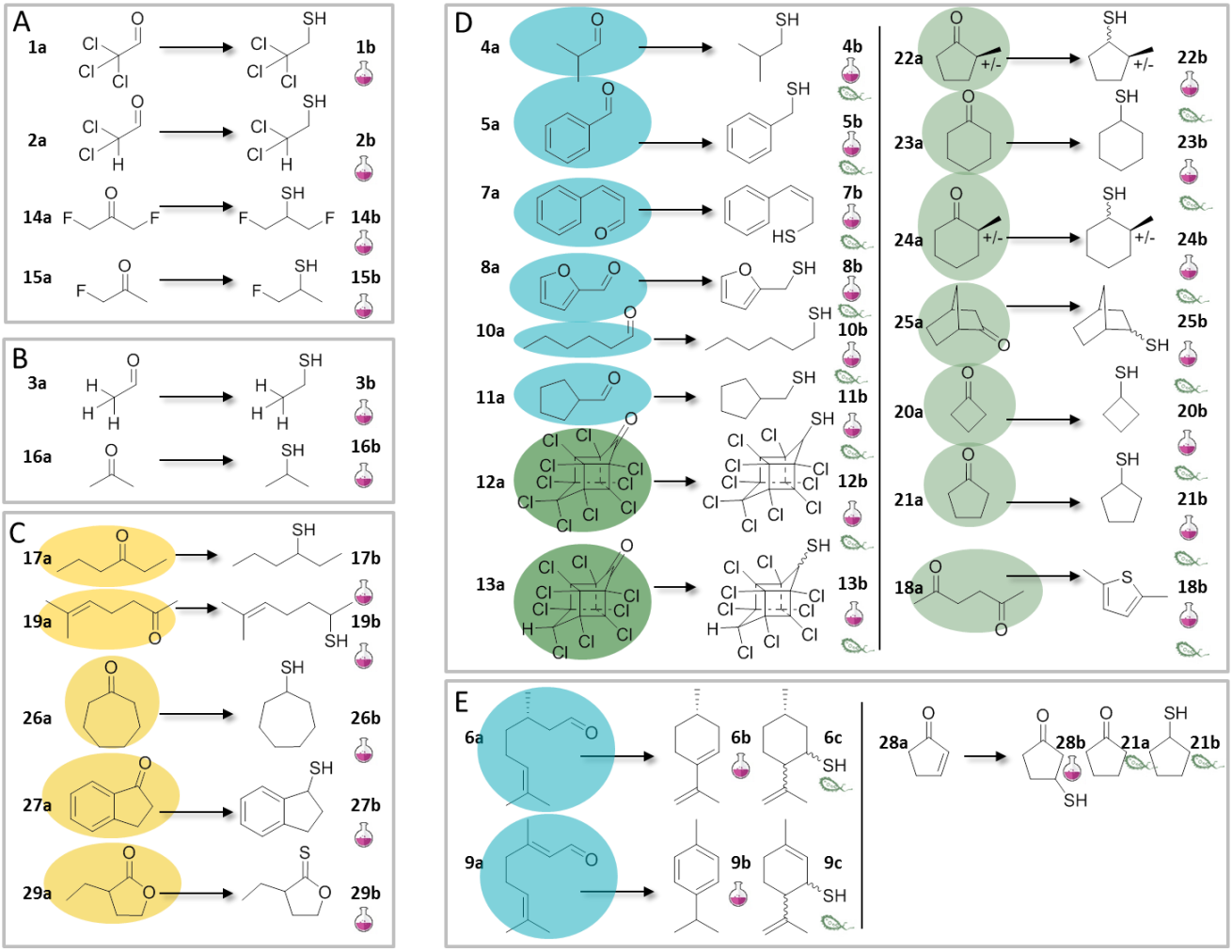


Figure 2. Description of the detected products obtained by bacterial and chemical reductive sulfidation. A-Carbonyl compounds not stable under bacterial conditions. B-Carbonyl compounds probably metabolized by Desulfovibrio sp.86. C-Ketones and lactone not transformed under bacterial conditions. D-Aldehydes and ketones that similarly react under chemical and bacterial conditions. E-Substrates that do not yield the same final products under chemical and bacterial conditions. The round-flask symbol represents products detected under chemical conditions, the green bacterium symbol is

associated with products detected under bacterial conditions. The colored circles correspond to the four clusters established to describe microbiological transformation.

To account for bacterial findings, we focused on 18 carbonyl substrates that showed the same reactivity toward chemical and microbiological transformation and discarded the others. Four categories were established according to the transformation degree of the carbonyl compounds: (i) not transformed (**17a**, **19a**, **26a**, **27a**), (ii) partially transformed (**20a-25a**), (iii) fully transformed after several weeks (**12a**, **13a**) and (iv) fully transformed after a few days (**4a-5a, 7a-8a, 10a-11a**).

As shown in Figure 3, group (a) consisting of chlordecone (**12a)** and 10-monohydrochlordecone (**13a**) had a specific behavior: they were completely transformed after 40 days by *Desulfovibrio* sp.86 into chlordecthiols **12b** and **13b** while the same products were predominantly formed after a simple treatment with P_4S_{10}. However, no other significant profile could be identified from the chemical and microbiological data. Indeed, pairing of groups (b), (c) and (d) did not lead to any statistical difference (Figure 3). From this set of data, it seems that the mechanism of chemical and bacterial reductive sulfidation may differ significantly.

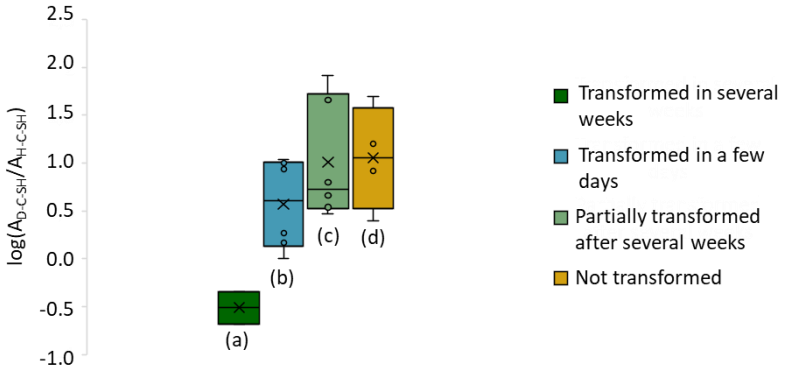
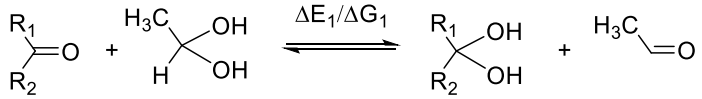


Figure 3. Boxplot comparing the reactivity of carbonyl compounds under chemical conditions ((i) P_4S_{10}; (ii) $NaBD_4$) and under microbiological conditions with *Desulfovibrio* sp.86. $A_{D\text{-}C\text{-}SH}/A_{H\text{-}C\text{-}SH}$ represents the peak area ratio of the deuterated thiol over the simple thiol formed from carbonyl substrates.

2.5. Calculation of structural, electronic and thermodynamic parameters

To account for the results of chemical and microbiological reductive sulfidation, we performed a series of quantum chemical calculations at the DFT level. We used the relative calculation approach to estimate the carbonyl hydration constants K_{hyd}[42]. K_{hyd} were computed from the Gibbs free energy differences of the exchange reaction, ΔG_1, and the thermodynamic definition of K_{hyd} (Scheme 1).

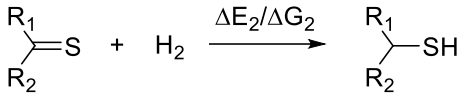


$$\log K_{hyd} = \log K_{hyd}(CH_3(H)CO) - \frac{\Delta G_1}{\ln 10\ RT}$$

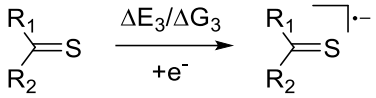
Scheme 1. Thermodynamic definition of the hydration constant K_{hyd}[42].

The exchange reaction includes acetaldehyde as the reference compound. Its K_{hyd} value is well-known experimentally ($\log K_{hyd}(CH_3(H)CO) = 0.03$)[43,44]. Numeric data of all these calculations are available in supplementary information (Tables S1-3). Additional parameters relative to the carbonyl moiety were calculated such as the LUMO energy level (lowest unoccupied molecular orbital), geometrical features and partial atomic charge (Table S5).

In the absence of detailed experimental information on the mechanisms of the bacterial and chemical reductive sulfidation reactions, two energy parameters have been calculated in order to estimate the ease of thione reduction, based on reactions displayed in Schemes 2 and 3.



Scheme 2. Hydrogenation of the thione.



Scheme 3. Electron affinity of the thione.

The Gibbs free energy of reaction showed in Scheme 3, ΔG_3, which corresponds to the electron affinity of the thione reactant, measures the stability of a monoelectronic reduced intermediate along the reduction process. ΔG_2 measures the thermodynamics of the full reduction process.

The LUMO energy level of the thione has been used as another parameter to evaluate the thione reducibility. Additional parameters such as the C=S● bond length d(C=S●) of the thioketyl radical anion, but also the spin density of the C=S● moiety were also calculated (Table S4).

2.6. Rationalization of carbonyl reactivity toward P_4S_{10} with selected calculated parameters

Here, we compared the ratio of GC-MS peak areas of deuterated thiols and non-deuterated thiols obtained by chemical reductive sulfidation with a selection of calculated parameters. For this part, data arising from 24 chemical experiments were exploited. The logarithm operator was applied to the ratio of GC-MS peak areas in order to minimize the effect of experimental fluctuations and mimic the relationship between the Gibbs free energy and the concentrations in the law of mass action. The logarithmic data set reflected at a semi-quantitative level the propensity of the thione intermediates to be easily reduced and thus made it possible to carry out correlation tests.

In view of the high level of hydration in chlordecone and congeners[25], we first examined the possible relationship between the hydration constant of the carbonyl substrates and their ability to be spontaneously reduced. Even if we could show a trend among the carbonyl compounds possessing electron-withdrawing substituents (Cl and F atoms, **1a**-**2a**, **12a**-**15a,** Figure S4) and known to be mainly present in the gem-diol form[25–28], this parameter could not explain the overall phenomenon. It means that the chemical reductive sulfidation is not very sensitive to the thermodynamics of the hydration reaction (Figure 4D). In addition, no specific relationship with any other selected parameters related to the carbonyl substrate could be detected (Table S4-5, Figure S4-5).

We then focused on the C=S bond reactivity. No trend could be established between the enthalpy of the C=S hydrogenation reaction and the chemical data set (Figure 4B). More interestingly, we found a correlation with the electron affinity of C=S (Figure 4A). In our case, the greater the energy of electron affinity was, the greater the proportion of thiol formed in the presence of P_4S_{10} became. As we could see, the substitution with electron-withdrawing groups including Cl and F atoms as well as the presence of aromatic rings conjugated with the thionyl moiety tends to increase this energy. It seems that the propensity of thiones to be reduced mono-electronically is the parameter driving their spontaneous reduction in the presence of P_4S_{10}. This assumption was supported by the significant relationship observed between the C=S● bond length d(C=S●) of the thioketyl radical anion and the set of experimental data (Figure 4C). Indeed, for some molecules, the added electron is trapped almost exclusively in the C=S bond, as revealed by the spin density on the C=S moiety which is greater than 0.9 (Table S4). In these cases, the elongation of the C=S bond upon reduction (0.09 to 0.1 Å) is strong as the electron is located in the π^* orbital. It corresponds to non-conjugated ketones and aldehydes deprived of electron-withdrawing substituents that did not show any spontaneous reduction. For conjugated molecules, the added electron is partially delocalized in the π system (lower C=S elongation, lower spin density on C=S), which stabilizes it more, resulting in a greater electron affinity. For chlorinated and fluorinated molecules, a part of the added electron goes to adjacent C-X bond (X = Cl or F). Again, this stabilizes the reduced form, and leads to a greater electron affinity. DFT calculations thus show that the presence of electron-withdrawing groups and/or conjugated systems plays a critical role in the stabilization of thioketylradicals, which thus appear to be the key intermediates in the spontaneous reduction of thiones.

All these observation are in agreement with what has been previously reported on cyclic and steroid ketones[12]. In their paper, the authors studied the propensity of thione intermediates to be spontaneously reduced using Na_2S and $LiAlD_4$. Finally, they managed to show that the reduction process was achieved via a single electron transfer mechanism by trapping the radical in an intramolecular cyclization reaction[12].

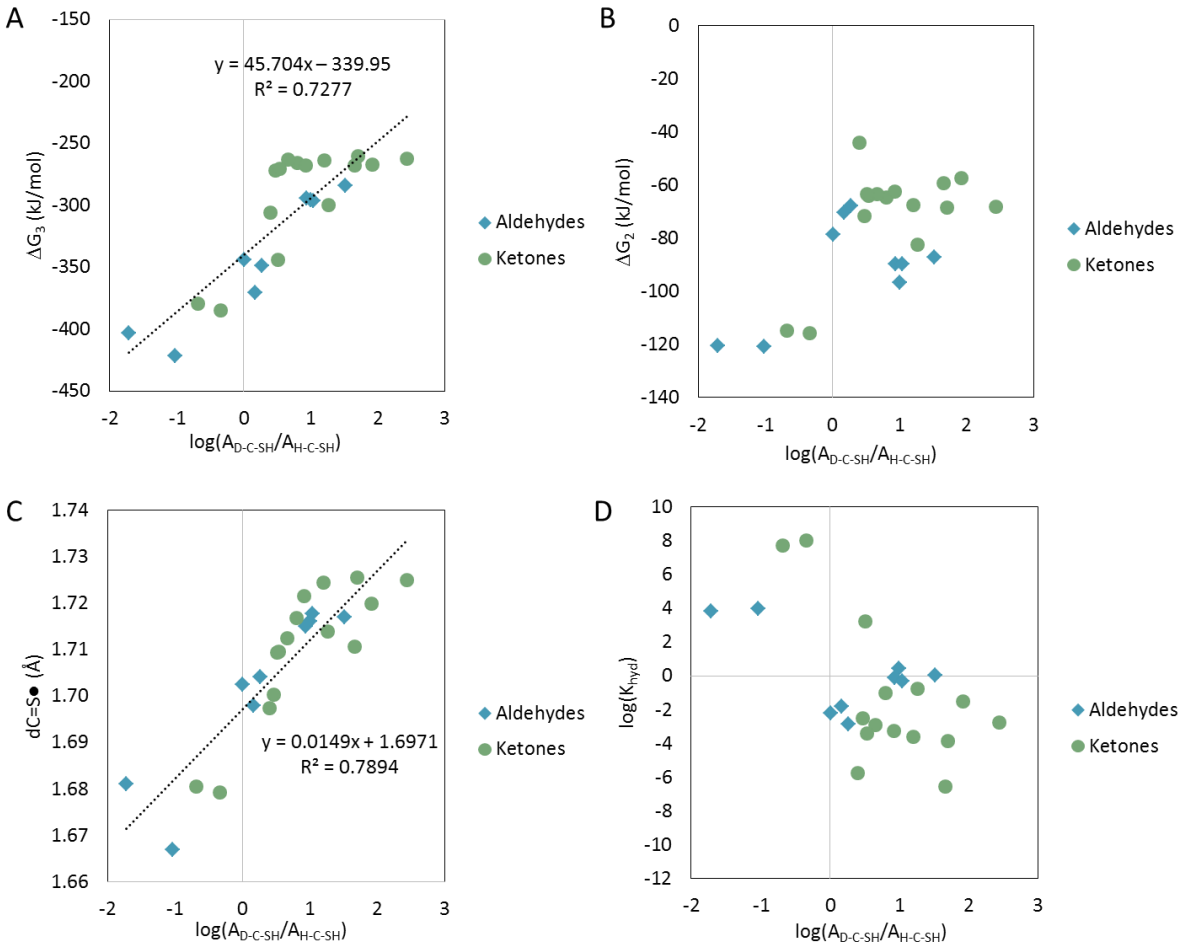
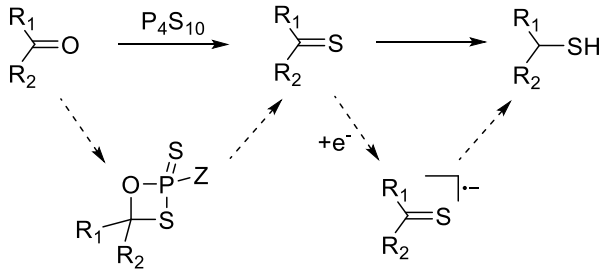


Figure 4. Correlations between the propensity of carbonyl compounds to undergo direct reductive sulfidation using P_4S_{10} and selected calculated parameters. A-electron affinity, B-Gibbs free energy for the hydrogenation reaction of C=S, C-the bond length of C=S●, D-log(K_{hyd}), the hydration constant of the C=O bond. $A_{D\text{-}C\text{-}SH}/A_{H\text{-}C\text{-}SH}$ represents the ratio of the GC-MS peak areas of the deuterated and the non-deuterated thiol.

On the basis of these results, we therefore proposed, the following sequence for the chemical reductive sulfidation without additional reductant (*e.g.* $NABH_4$ or $LiAlH_4$): (i) P_4S_{10} initially reacted with carbonyl compounds to form four-membered ring intermediates which decomposed into the corresponding thiones as previously assumed[10]; (ii) then, radical and/or reducing species such as H_2S present in the reaction mixture reacted with these thiones via a single electron transfer mechanism; (iii) finally, the protonation of the resulting C=S● radical led to the observed thiols (Scheme 4). For the carbonyl compounds involved in a conjugated system or possessing electron-withdrawing groups, the electron would be partially delocalized in the π-system or in the C-X bond, which would highly enhance the reduction of the thione intermediates.



Scheme 4. Proposed pathway for the chemical reductive sulfidation in the presence of P_4S_{10}.

Substrate	Theoretical parameters			Chemical results		Microbiological results		
	ΔG_3 (kJ/mol)	ΔG_2 (kJ/mol)	Log(K_{hyd})	log($A_{D\text{-}C\text{-}SH}/A_{H\text{-}C\text{-}SH}$)	de (%)	Transformation state	de (%)	Incubation time (days)
1a	-422.1	-120.8	3.81	-1.03		d		/
2a	-403.9	-120.5	3.98	-1.72		d		/
12a	-388.2	-115.7	8.03	-0.34		Fully transformed		40
13a	-380.0	-115.0	7.69	-0.68	13	Fully transformed	30	40
7a	-370.7	-70.5	-1.79	0.17		Fully transformed		10
14a	-344.7	-63.5	3.21	0.51		d		/
15a	-344.6	-78.8	-2.22	0.01		Fully transformed		3
8a	-349.4	-67.9	-2.88	0.27		Fully transformed		3
9a	-328.6	-76.7	-3.62	/		Fully transformed		3
27a	-306.1	-44.1	-5.75	0.4		Not transformed		/
15a	-300.0	-82.41	-0.78	1.26		d		/
3a	-284.6	-87.4	0.03	1.52		m		/
10a	-296.4	-96.9	0.41	1.10		d		/
4a	-294.5	-89.7	-0.10	0.94		Fully transformed		3
11a	-296.9	-89.8	-0.30	1.04		Fully transformed		10
6a	-292.7	-92.8	-1.73	/		Fully transformed		3
20a	-272.0	-71.8	-2.50	0.47		Partially transformed		/
21a	-270.9	-64.2	-3.41	0.54		Partially transformed		/
26a	-268.1	-62.6	-3.27	0.92		Not transformed		/
22a	-268.0	-59.3	-6.54	1.66	19	Partially transformed	70	/
24a	-267.4	-57.3	-1.53	1.92	44	Partially transformed	95	/
24a	-266.5	-64.6	-1.00	0.80		Partially transformed		/
18a	-288.7	-67.7	-3.51	/		Partially transformed		/
16a	-262.8	-68.3	-2.77	2.44		m		/
19a	-264.1	-67.7	-3.63	1.20		Not transformed		/
25a	-263.5	-63.5	-2.89	0.66	15	Partially transformed	76	/
17a	-260.6	-68.5	-3.84	1.70		Not transformed		/
29a	-225.7	-16.3	-9.27	/		Not transformed		/

Table 1. Summary of the main theoretical, microbiological and chemical results concerning the reductive sulfidation reaction. $A_{D\text{-}C\text{-}SH}/A_{H\text{-}C\text{-}SH}$ represents the GC-MS peak area ratio of the deuterated thiol over the non-deuterated thiol. de stands for diastereomeric excess. Incubation time indicates the time required to observe a complete disapearance of the target carbonyl compounds by GC-MS analysis. The transformation state only refers to the transformation of the carbonyl into the target thiol (d and m stand for decomposition and metabolization, respectively).

2.7. Influence of molecular parameters on bacterial reductive sulfidation of carbonyls

In contrast to the chemical results, no (semi-)quantitative data could be retrieved from the microbiological experiments. We therefore decided to exploit the clustering previously introduced with four levels of transformation, varying from level 1 (no transformation) to level 4 (complete transformation within several days). Contrary to previously, four molecules were added to these groups: **6a** and **9a** to cluster (b^+), **18a** to cluster (c^+) and **29a** to cluster (d^+). We applied statistical tests (ANOVA) to assess the significant differences between the clusters for each selected parameter. Since cluster (a) included only two compounds (chlordecone **12a** and 10-monohydrochlordecone **13a**), the statistical tool was not applicable in this case.

We first compared the different clusters with respect to the hydration constant of carbonyl substrates. As in the case of chemical reactions, we could not observe any clear influence of this parameter on the level of the observed biotransformation (Figure 5C). The SOMO and LUMO energies of the thiones, the bond length of C=S as well as the C=S and HC-SH radicals did not correlate better with the microbiological results (Table S4, Figure S6). However, the calculation of the partial charge of the oxygen atom on

the C=O bond enabled to show a slight distinction between groups (c^+) and (d^+) (Figure 5D). With respect to the electron affinity of the C=S bond and the Gibbs free energy of the C=S hydrogenation, we found significant differences between clusters (b^+) and (c^+) and also between clusters (b^+) and (d^+) (Figures 5A and 5B). Even so, among the selected parameters, none of them could discriminate between groups (c^+) and (d^+). It is noteworthy that the previous papers describing bacterial reductive sulfidation[22,23] never mentioned any trace of the possible thione intermediate just as in our case. It may be the reason why these parameters focusing on the C=S bond did not correlate well with the qualitative trend presently observed.

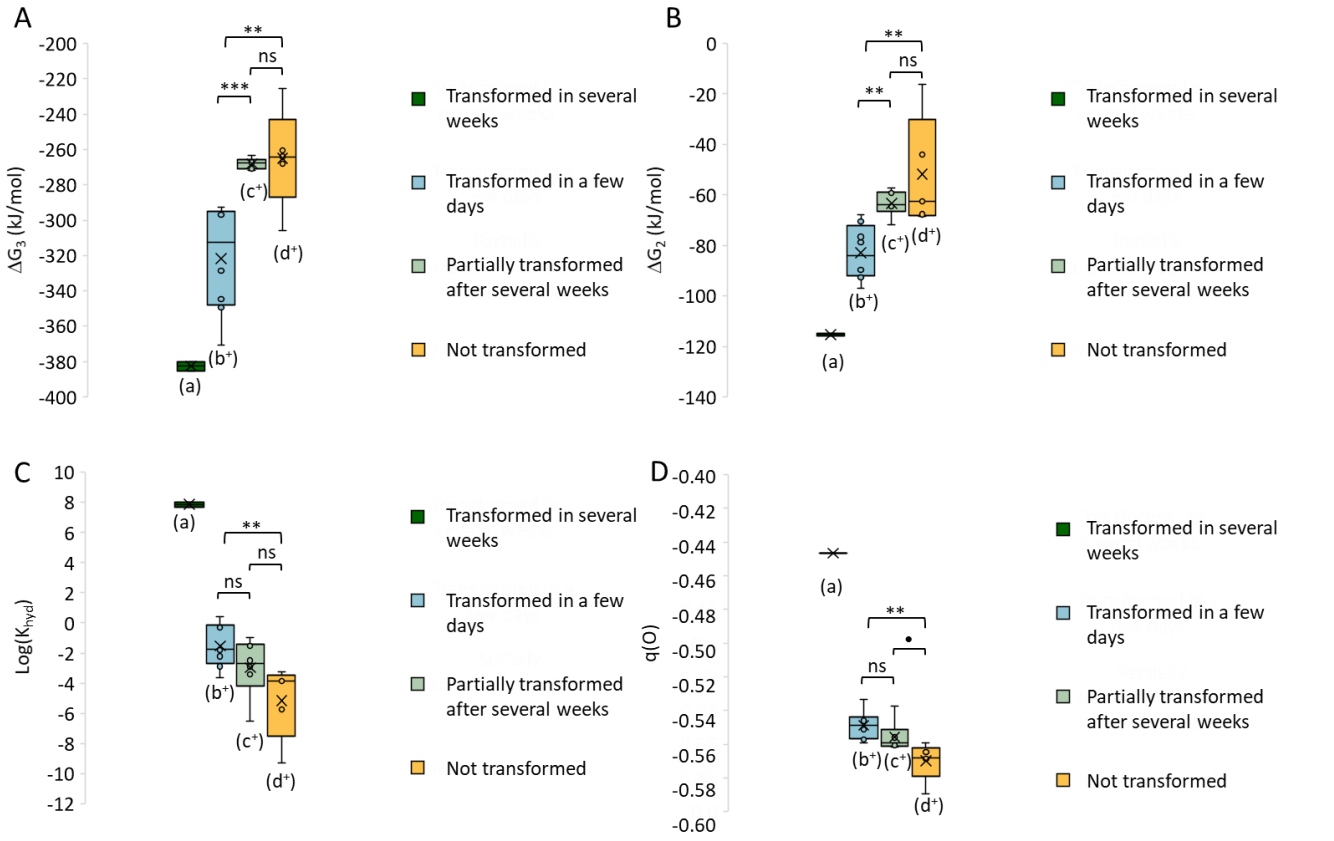


Figure 5. Boxplots displaying the different transformation states of the carbonyl compounds after incubation with *Desulfovibrio* sp.86 as functions of selected calculated parameters. **A-electron affinity, B- Gibbs free energy of C=S hydrogenation, C-log(K_{hyd}), the hydration constant of the C=O bond, D-partial charge of the oxygen atom on the C=O bond.• = 0.1 < p-value < 0.05, * = 0.05 < p-value < 0.01, ** = 0.01 < p-value < 0.001, *** = 0.001 < p-value < 0, ns = not significant.**

Interestingly, we could correlate the biotransformation of groups (a) and (b^+) to *Desulfovibrio* sp.86 growth. Indeed, while a couple of days were needed to achieve complete transformation of aldehydes, several weeks were required to fully convert chlordecone (**12a**) and 10-monohydrochlordecone (**13a**) into their thiol derivatives (Figure 6). As can be seen in Figure 6C-H, the reductive sulfidation of the aldehydes was correlated with the growth phase, whereas in Figure 6A-B, the biotransformation of group (a) occurred mainly during the stationary phase. These differences could be explained by the very low solubility of chlordecone (**12a**) and 10-monohydrochlordecone (**13a**) in water, estimated around 2 mg/L at pH 7 and 25°C[45], and/or by the involvement of two distinct mechanisms depending on the nature of the substrate.

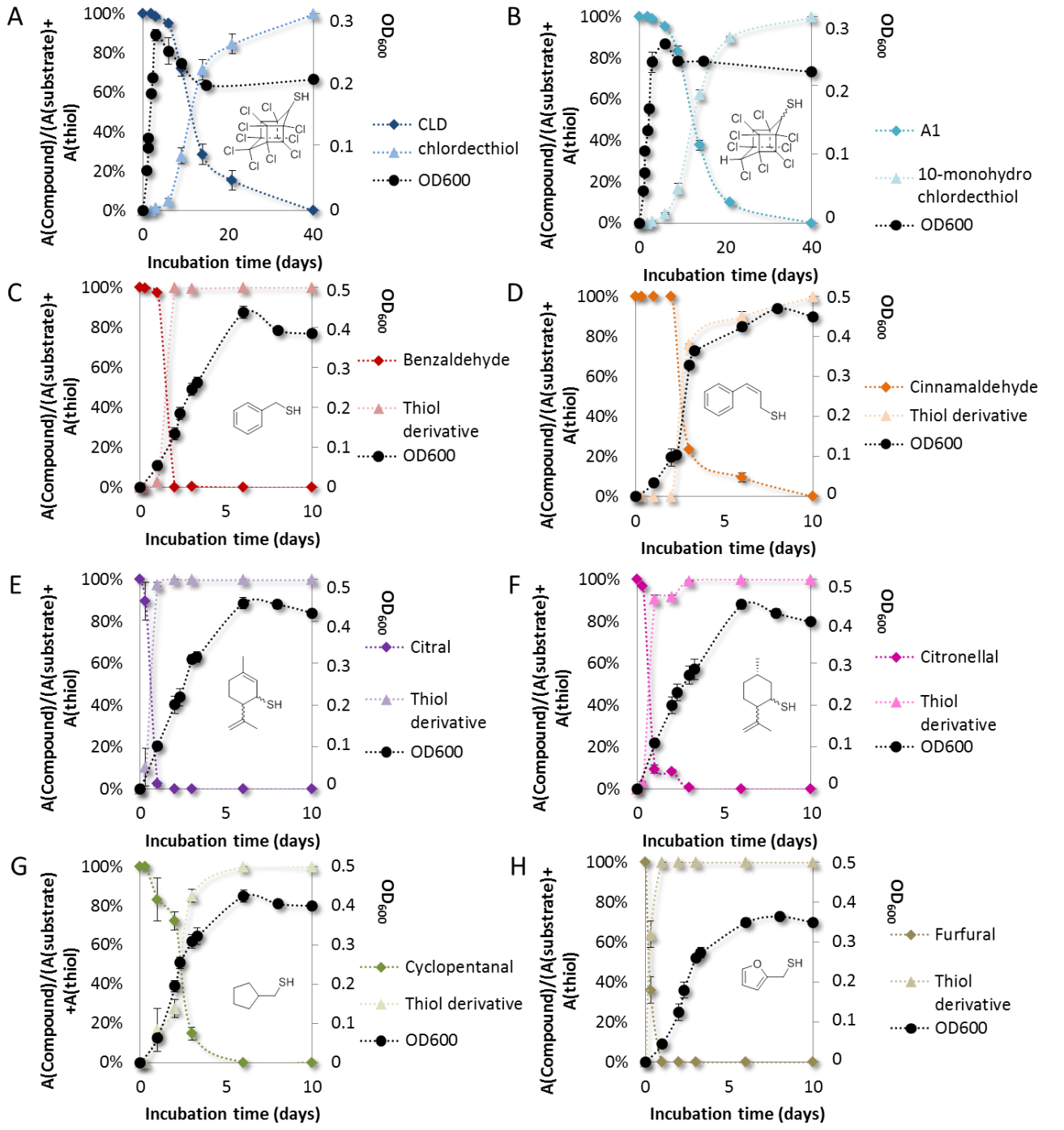


Figure 6. Reductive sulfidation of selected carbonyl compounds, over time, by *Desulfovibrio* sp.86, in MMD medium and CA conditions, monitored by GC-MS analysis. A-Chlordecone transformation[16], B-10-monohydrochlordecone transformation, C-Benzaldehyde transformation, D-Cinnamaldehyde transformation, E-Citral transformation, F-Citronellal transformation, G-Cyclopentanal transformation and H-Furfural transformation. OD_{600} corresponds to the optical density at 600 nm. A corresponds to the GC-MS peak area.

3. CONCLUSION

Bacterial reductive sulfidation has been rarely documented, although it represents an efficient strategy for the formation of thiols. In the present work, we studied the substrate-versatility of the reductive sulfidation activity of *Desulfovibrio* sp.86 and compared it to a two-step chemical procedure using the classical sulfur donor P_4S_{10}. We showed that *Desulfovibrio* sp.86 transforms a variety of substrates (most preferably aldehydes or ketones substituted with electron-withdrawing groups and/or included in a ring system). Notable differences compared to chemical reductive sulfidation offer new perspectives for the selective synthesis of thiolated compounds.

A number of target thiols were partially or completely formed in the presence of P_4S_{10} without the additional reduction step required according to the literature. Several parameters (electron affinity of the C=S bond, length and spin-density of the C=S moiety of the thioketyl radical anion) obtained by quantum chemical calculation rationalize these observations. Indeed, DFT results demonstrate that the presence of electron-withdrawing groups and/or aromatic rings which experimentally promote the reductive sulfidation can stabilize the thioketyl radical through partial delocalization of the single electron over sigma and/or pi bonds. We therefore concluded that a single-electron transfer to the thione intermediate was likely to occur. The same correlation was not observed for the bacterial reductive sulfidation. Its mechanism, whether or not involving the thione, may differ according to the type of substrates (aldehydes or ketones).

4. EXPERIMENTAL SECTION

4.1 Chemicals and media

Chlordecone was obtained from Azur Isotopes (purity 98%). Na_2S (≥ 98%), phosphorus pentasulfide (99%), aldehydes, ketones and lactone were purchased from Sigma Aldrich. Acetonitrile (MeCN, LC-HRMS grade) and acetone (> 99.9%) were obtained from VWR Chemicals. The chemicals used for microbiological media were obtained from Sigma Aldrich.

4.2 Anoxic microbial incubations

The anoxic incubation conditions used for *Desulfovibrio* sp.86 have already been described[16]. They were carried out in MMD medium consisting of the MM enriched mineral medium previously described[16] supplemented with lactate as carbon source (50 mM), yeast extract (1 g/L), Na_2SO_4 (25 mM) and Na_2S as reducing agent (0.4 g/L). *Desulfovibrio* sp.86 cultures were incubated at 30°C, in an oven, under anaerobic conditions (N_2/H_2 (98/2; V/V)). They were carried out in sealed culture vials, closed with butyl rubber septa. *Desulfovibrio* sp.86 cultures were inoculated at the onset of an experiment with 0.5 mL pre-culture pre-grown in the oven for 24 hours.

All microbiological experiments were performed in 100 mL vials filled with 50 mL of MMD inoculated with an actively growing *Desulfovibrio* sp.86-1 culture (1/100 v/v) in duplicate and an abiotic control was included. Carbonyl compounds were added to a final concentration of 40 mg/L in each vial.

4.3 Extraction/sampling for microbiological culture monitoring

Chlordecone and 10-monohydrochlordecone transformations were monitored by GC-MS analysis. Benzaldehyde, cinnamaldehyde and indan-1-one transformations were monitored by GC-MS and HS-GC-MS analyses. The other carbonyl compounds transformations were recorded by HS-GC-MS analysis.

For GC-MS analysis, 500 µL of the liquid medium were collected and extracted twice using 250 µL of isooctane. The combined organic layers were then analyzed by GC-MS analysis.

When HS-GC-MS was required, 1 mL of cultures was sampled and put into a Chromacol 10-HSV vial of 10 mL (Agilent). The headspace gas was then analyzed.

4.4 Analytics

GC-MS analyses were carried out using a Thermo Fisher Focus GC coupled to a single-quadrupole mass spectrometer (Thermo Fisher DSQ II). The instrument was equipped with a non-polar 30 m × 0.25 mm × 0.25 µm DB-5MS column (Agilent J&W) and a split/splitless injector. Ionization conditions and GC program used for monitoring chlordecone and 10-monohydrochlordecone were described elsewhere[46] (method GC-MS-1).

For benzaldehyde, cinnamaldehyde and indan-1-one, the GC program started at 30°C (hold time 6 min), continued with 15°C/min to 90°C (hold time 8 min), followed by 10°C min^{-1} to 200°C (hold time 1 min) (method GC-MS-2).

HS-GC-MS analyses were performed on a Thermo Fisher Trace 1300 coupled to ISQ 7000 VPI single quadrupole mass spectrometer. The instrument was equipped with a 30 m × 0.25 mm × 0.25 µm DB-624-UI column (Agilent J&W), a split/splitless injector and an automatic sampler TriPlus RSH coupled to a Headspace tool. For mass spectrometry (MS) analyses, the following standard working conditions were applied: electronic impact ionization, positive mode detection, ion source at 220°C, detector voltage 70 eV, full scan mode m/z 33–300 (scan time 0.20 sec). The temperatures of the injection and transfer lines were set at 200°C, respectively 280°C. After the incubation time, 1 mL of the headpace gas was injected each time with a filling speed of 10 min/mL, an injection speed of 10 mL/min and a penetration speed of 10 mL/s.

For acetone, ethanal and 1-fluoroacetone monitoring, vials were incubated for 1 min at 40°C and sampled with a syringe at 40°C. The split mode was applied (30°C, flow rate at 16.7 mL/min, with a ratio of 33.4). The carrier gas was helium at 0.5 mL/min for 1 min followed by a gradient of 0.05 mL/min until reaching 1 mL/min (hold time 9 min). The GC program was isocratic at 24°C for 20 min (method HS-GC-MS-1).

For the other carbonyl compounds, monitoring vials were incubated for 5 min at 50°C and sampled with a syringe at 50°C. The splitless mode was applied at 150°C.The carrier gas was helium at a constant flow rate of 0.5 mL/min. The GC program started at 30°C (hold time 6 min), continued with 15°C/min to 130°C (hold time 0.5 min), followed by 7°C min^{-1} to 250°C (hold time 10 min) (method HS-GC-MS-2).

4.5 Chemical reductive sulfidation of carbonyl compounds

Phosphorus pentasulfide (20 mg, 4.5 10^{-5} mol, 4.5 eq) was added to a solution of carbonyl compound (1.0 10^{-5} mol, 1 eq) in acetonitrile (1 mL). The reaction mixture was stirred under N_2, at room temperature for 12 hours. Then 500 µL of methanol containing $NaBD_4$ at 1.0 10^{-1} M was added (5.0 10^{-5} mol, 5 eq), and was stirred for 12 hours at room temperature.

4.6 DFT calculations

Calculations were carried out with the Gaussian09 package[52]**.** All structures were fully optimized without any symmetry constraints at the DFT level by means of the PBE0[47,48] (PBE1PBE keyword in G09) and M06-2X[49] functionals. The 6-31+G(d,p) basis set was applied for all atoms for geometry optimization. Geometries have been first optimized in the gas phase at the PBE0/6-31+G(d,p) and M06-2X/6-31+G(d,p) levels. In order to ensure that solvation has almost no influence on the geometry, the geometries were re-optimized at the PBE0/6-31+G(d,p) level and including a continuum solvation method (integral equation formalism version of the polarizable continuum model (IEFPCM) for water). Each stationary point has been characterized with frequency analysis and shows no negative eigenvalues, as required for local minimum. Final energy calculations at the PBE0 and M06-2X levels associated with the 6-311++G(2d,2p) basis set, both in the gas phase and including solvation effect, have been achieved on the optimized geometries. To get accurate geometries and energies, the SCF convergence criterion was systematically tightened to 10^{-8} au, and the force minimizations were carried out until the rms force became smaller that (at least) 1 x 10^{-5} au ("tight" optimization keyword in Gaussian 09). The "UltraFine" grid (99 radial shells and 590 angular points per shell) was used throughout the calculations, as recommended when using Gaussian 09. Unrestricted formalism (UDFT) was used for radicals.

The results obtained show an excellent agreement between PBE0 and M06-2X data and a minor influence of the implicit solvation on the optimized geometries (Figure S2). Comparison of the DFT results with post-HF CCSD(T) calculations (Figure S1) does not reveal any particular deficiencies in the functionals used, even in the case of the study of radicals[50]. Furthermore, comparison with experimental data (Figure S3) shows a slightly better agreement for M06-2X data. Therefore, only M06-2X data are presented in the text. The Gibbs free energies presented in this article are thus IEFPCM(water)-M06-2X/6-311++G(2d,2p)//M06-2X/6-31+G(d,p) electronic energies (which include solvation-energy corrections from the IEFPCM method) modified with thermal and entropy corrections from M06-2X/6-31+G(d,p) calculations.

4.7 Statistics

Statistical tests have been performed using RStudio, according to the following methods: normality was checked with a Shapiro test. If the distribution was normal, the homogeneity of variances was checked with a Bartlett test and a two-way ANOVA test was performed. If the distribution was not normal, a non-parametric test coming from the ARTool package[51] was used. Tukey analysis was subsequently used as a post-hoc analysis.

5. ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge at

General procedure for the identification of compounds using GC-MS analysis (Table S6), extra details about DFT calculations (Figures S1-S3, Tables S1-S5), extra correlations between chemical and microbiological experiments and theoretical parameters (Figures S4-S6), chromatograms and mass spectra of chemical and microbiological reductive sulfidation (Figures S7-S52) (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mails: plsaaidi@genoscope.cns.fr, gilles.frison@sorbonne-universite.fr, denis@genoscope.cns.fr

ORCID

Sébastien Chaussonnerie: 0000-0001-6065-1348

Oriane Della-Negra: 0000-0002-9508-2445

Gilles Frison: 0000-0002-5677-3569

Denis Le Paslier: 0000-0003-4335-9956

Pierre-Loïc Saaidi: 0000-0003-3028-4764

Present Addresses

[†]Génomique Métabolique, Genoscope, Institut François Jacob, CEA, CNRS, Univ Evry, Université Paris-Saclay, 91057 Evry, France

[‡]Laboratoire de Chimie Moléculaire, Ecole Polytechnique, CNRS, IP Paris, Palaiseau, France

[ǂ]Sorbonne Université, CNRS, Laboratoire de Chimie Théorique, Paris, France

Author Contributions

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Notes

EC numbers were checked using Brenda database (www.brenda-enzymes.org).
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ABBREVIATIONS

de, diastereomeric excess, DFT, Density Functional Theory; EC, Enzyme Commission; GC-MS, Gas Chromatography coupled to Mass Spectrometry; HS, Headspace; hyd, hydration, LUMO, Lowest Unoccupied Molecular Orbital; MM, Mineral Medium; OD, Optical Density; RS, Reductive Sulfidation; SOMO, Singly Occupied Molecular Orbital.

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