Microbially derived P=S and P=Se bond formation

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Abstract

Microbial metabolism is a diverse and sustainable source of synthetic reagents that can be programmed for controlled and high-level production via synthetic biology. However, despite the chemical diversity of metabolism, the chemical utility of metabolites, and the available tools to control metabolic chemistry, there remain few examples of the use of cellular metabolites directly for chemical synthesis. Herein we report that diverse bacteria perform P=S bond formation (Ph_3P to Ph_3PS) via central sulfur metabolism and non-enzymatic chemistry *in vivo* and can also be applied to effect microbial P=Se bond formation (Ph_3PSe). To the best of our knowledge, this is the first biochemical and genetic investigation of P=S bond formation in a microbial cell and the first use of microbial metabolites for P=Se bond formation in chemical synthesis.

Introduction

In nature, main group sp^2-sp^2 bonds are limited to those formed during photosynthesis (O_2), biosynthesis (C=N, P=O, S=O and N=N), or broken during assimilatory metabolism (N_2 and SO_2) and along respiratory electron transport chains (O_2, S=O and NOR). However, the formation of similar sp^2-sp^2 bonds between phosphorus and sulfur (P=S) has not readily been observed in biology. In chemistry, P=S bonds are found in synthetic reagents used for episulfide or thioketene formation[1] and within phosphorothiolate backbones of nucleotide drug candidates to protect against metabolic degradation *in vivo*[2,3]. The lack of established methods to form P=S using biological tools means the functional transformation remains only accessible by chemical synthesis. Existing methods for this largely rely on the rection of P(III) substrates with elemental or electrophilic sulfur reagents in organic solvent under abiotic conditions[4]. This is despite the wealth of electrophilic $S^{\delta+}$ metabolites that exist in biological systems which can be intercepted using biocompatible chemistry or overproduced for delivery into existing reactions using synthetic biology. Herein we report the discovery that diverse microorganisms can be used to perform P=S and P=Se bond formation by interfacing non-enzymatic R_3P oxidation with native sulfur metabolic pathways *in vivo* and apply this to the synthesis of triphenylphosphine sulfide and triphenylphosphine selenide.

Results and Discussion

Discovery of these unusual microbial reactions began by screening diverse, chemically uncharacterised bacteria from the National Collection of Industrial, Food and Marine Bacteria (NCIMB) for the ability to modify P-containing small molecules. A panel of 35 species were curated from a range of environments with different culturing conditions and varying degrees of established literature (Fig. 1A and Table S1). Small molecule targets were limited to triphenylphosphine (Ph_3P) and triphenylphosphine oxide (Ph_3PO). Whilst Ph_3PO is considered a waste product, modification presents an interesting opportunity to regenerate Ph_3P for further industrial scale use in various named synthesis reactions[5] or produce other useful phosphines and phospholes[6]. For screening, cultures were incubated

to accumulate significant biomass (OD_{600} >0.4), after which phosphines were added at 3 mM under aerobic and microaerobic conditions then incubated for 44 hours (220 rpm). Cultures were extracted with organic solvent then analysed by ^{31}P NMR.

Although no NMR signal changes were observed in any Ph_3PO cultures (Fig. S3-S5), a small additional downfield peak at δ +43.40 ppm was observed in 11 different species in addition to <5% Ph_3PO autoxidation (Fig. 2A and Fig. S6-S9). Such a large de-shielded shift in ^{31}P resonance from δ -5.39 ppm to δ +43.40 ppm suggested direct modification of the P(III) atom by a more electronegative group. Considering established P(V) main group chemistry literature, the peak was identified as triphenylphosphine sulfide (Ph_3PS)[7]. Intrigued by this modification, we sought to understand it further; however, many screened species lacked detailed metabolic characterisation and annotated genomes which presented challenges to identifying the reason(s) why the bacteria could mediate this chemistry.

Phylogenetic analysis showed P=S bond formation was spread throughout the phyla and suggested this transformation could be completed by *Escherichia coli.* Indeed, we found *E. coli* BW25113 could facilitate Ph_3PS formation under identical reaction conditions (Fig. 2B and Fig. S10) and moved to identify the metabolic sulfur source enabling the reaction. Bacterial sulfur metabolism is diverse, multiplexed, and highly regulated but can ultimately be simplified to the biochemical reactions centring around the interplay of cysteine metabolism (Supplementary Figure S11). Based on this understanding, *E. coli* cultures were supplemented with either 50 mM L-Cys or L-Met or 25 mM L-Cys_2 or L-Sec_2 as a primary sulfur and selenium source. Whilst L-Met had no effect on Ph_3PS conversion, L-Cys and L-Cys_2 resulted in a 3.1- and 7.4-fold increase respectively (Fig. 3A). The addition of L-Sec_2 also resulted in another additional peak at δ +35.29 corresponding to triphenylphosphine selenide as indicated by comparison to literature[8] and detection of a large 720 Hz $^1J_{P\text{-}Se}$ coupling in the 1D ^{31}P NMR spectrum (Fig. S2). Live cells were required for product formation as the addition of L-Cys_2 to LB media in the absence of cells did not increase conversion to Ph_3PS.

To further investigate the source of metabolic sulfur, we screened 11 KEIO *E. coli* knockout strains to the L-Cys screen. These knockouts examined key points in enzymatic persulfide (enzyme-SSH) biochemistry either directly by targeting persulfide-generating desulfurases (Δ*iscS*, Δ*ynjE*, Δ*sufS*, Δ*csdA*) and sulfur transferases (Δ*thiI*, Δ*tusA,* Δ*sseA,* and Δ*pspE*), or indirectly by targeting regulators (Δ*iscR*) and transport proteins (Δ*tcy* and Δ*eamA*). By restricting persulfide generation, we anticipated knockouts would reduce Ph_3PS conversion by reducing the amount of reactive sulfur. Interestingly, no knockout eliminated the increased conversion entirely; however, disrupting cysteine import and sulfur export resulted in a decreased conversion by 57% and 59%, respectively (Fig. 3B). Whilst unsurprising given functional overlap in sulfur metabolism, these combined experiments clearly implicated the role of L-Cys in P=S formation and that this likely occurs externally to the cell.

The major metabolic products from L-Cys utilisation and degradation is H_2S[9,10] which, through reactions with O_2, can generate sulfane sulfurs via SO_2 under mild conditions. As such, we hypothesised that enzymatically derived H_2S may serve as the missing bridge between biological processes and abiotic chemistry. By adding Ph_3P to spent culture supernatant isolated from cells grown under analogous conditions in the presence of L-Cys, we observed increased Ph_3PS formation (0.17 mM), however this was reduced compared to actively growing *E. coli* cultures (0.25 mM; Fig. 4). Inversely, minimal product conversion was detected when the washed cell pellet was incubated with Ph_3P, confirming the reactive sulfur source was secreted from the cells. By replacing dissolved and headspace gas reaction prior to the addition of Ph_3P, Ph_3PS conversion was reduced by 58%. This indicated H_2S forms a key

sulfur source that can either react with Ph_3P directly under the culture conditions or via sufane sulfur formed by H_2S oxidation during the reaction. Although it is interesting that not all the original species from the NCIMB curation shown to produce Ph_3PS are known to produce H_2S, sulfane sulfur remains a key intermediate in [Fe-S] clusters[11] and tRNA synthesis[12,13] which are ubiquitous in biology thus we hypothesise elevated concentrations of such sulfur sources are responsible for the detectable conversion observed in these strains.

Conclusions

In conclusion, diverse bacteria have been found to promote non-enzymatic P=S bond formation *in vivo* through the generation of reactive sulfur metabolites derived from L-Cys. This microbial chemistry was found in 11 distinct species within an uncharacterised national culture collection and can also be observed and optimised to occur in preparative yields using laboratory strains of *Escherichia coli*. Genetic and biochemical studies indicate the source of reactive sulfur is multifaceted and likely stems from metabolic H_2S formation and sulfane sulfur generation under aerobic conditions, followed by abiotic P=S bond formation. This principle can also be applied to form P=Se bonds *in vivo* when *E. coli* is provided L-Sec_2. Overall, this work highlights the diverse metabolic chemistry of microorganisms and how microbial metabolism can be coerced and applied to create new synthetic methods for sustainable chemical synthesis.

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Contributions

C.L.T: conceptualisation, microbial curation, experimental, data analysis, troubleshooting. S.W: conceptualisation, data analysis, troubleshooting. S.L: conceptualisation, microbial curation, troubleshooting. R.G: NMR submission for NCIMB screen. The manuscript was written and edited by S.W and C.L.T.

Competing Interests

Dr Samantha Law is an employee of NCIMB Ltd. and may possess competing financial and non-financial interests.

Data Availability

The data supporting this article have been included as part of the Supplementary Information.

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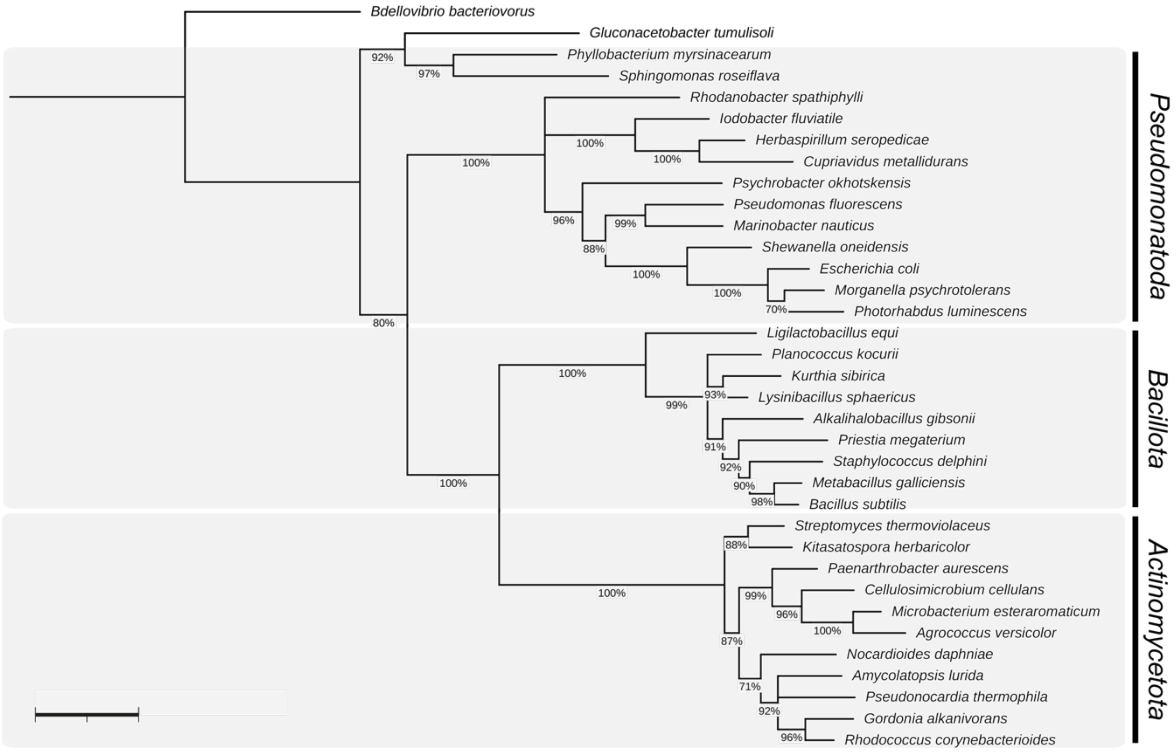
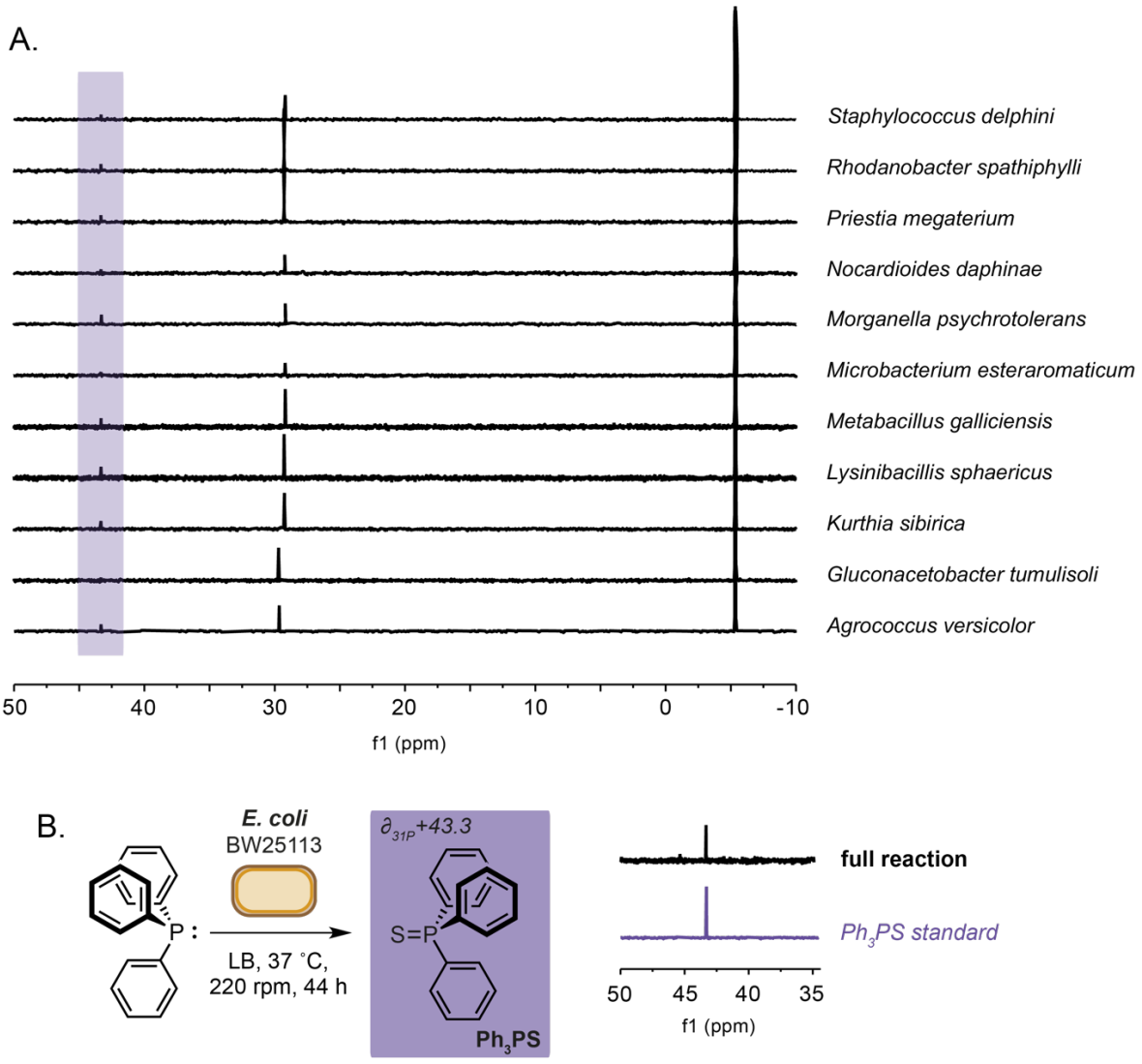


Figure 1. Maximum Likelihood tree showing the phylogenetic diversity of microorganisms used for screening. Tree was constructed using the General Time Reversible (GTR) substitution model with gamma distributed rates (+G) and invariable sites (+I) and shows consensus of nodes with >65% bootstrap support based upon 500 replicates. *Bdellovibrio bacteriovorus* was used as an outgroup to infer genetic distance. Bar represents 0.1 substitutions per nucleotide position.



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154 **Figure 2.** A) ^{31}P NMR spectra from extracts of microbial cultures incubated in the presence of
155 triphenylphosphine. B) ^{31}P NMR spectra from a culture of *E. coli* BW25113 incubated in the presence
156 of triphenylphosphine. Reaction conditions: Ph_3P (3 mM) added to culture of *E. coli*
157 BW25113_pET28a(+) (OD_{600}= 0.4-0.6) in LB media and incubated at 37 ˚C (200 rpm) for 44 h before
158 extraction using ethyl acetate and analysis by ^{31}P NMR.

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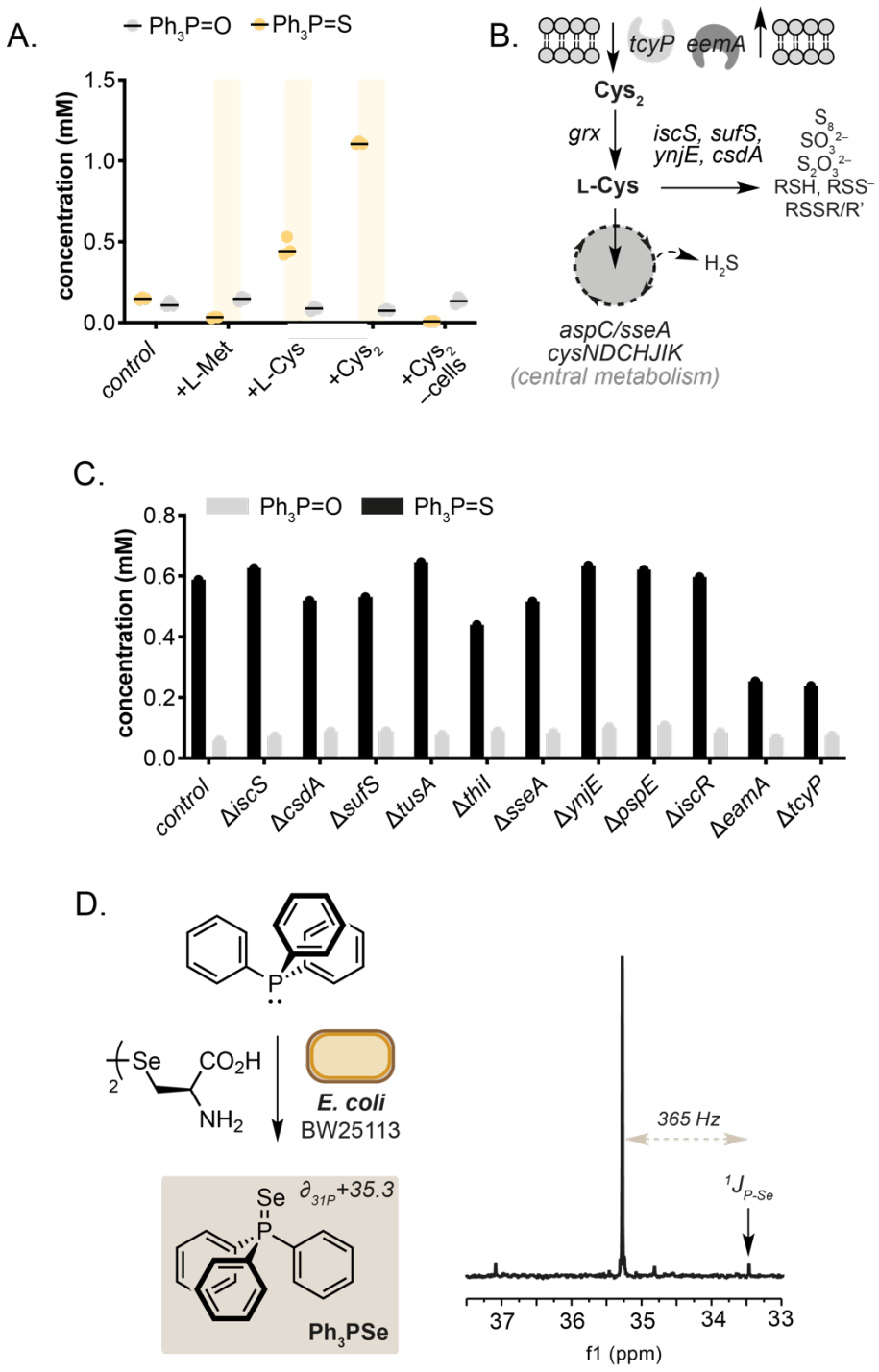

Figure 4. A) Amino acid dependent TPPS formation by *E. coli*. B) Key sulfur metabolism pathways in *Escherichia sp.*. C) Effect of sulfur metabolism gene knockouts on Ph_3PS formation by *E. coli* BW25113. C) Ph_3PSe formation by *E. coli* BW25113 incubated in the presence of L-Sec and triphenylphosphine detected by ^{31}P NMR. A 720 Hz $^{1}J_{31P\text{-}77Se}$ coupling is also observed confirming P=Se bond formation. Reaction conditions: L-Sec (25 mM) added to culture of *E. coli* BW25113_pET28a(+) (OD_{600}= 0.4-0.6) in LB media and incubated at 37 ˚C (200 rpm) for 44 h before extraction using ethyl acetate and analysis by ^{31}P NMR.