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Generation of lysolipin derivatives by genetic engineering

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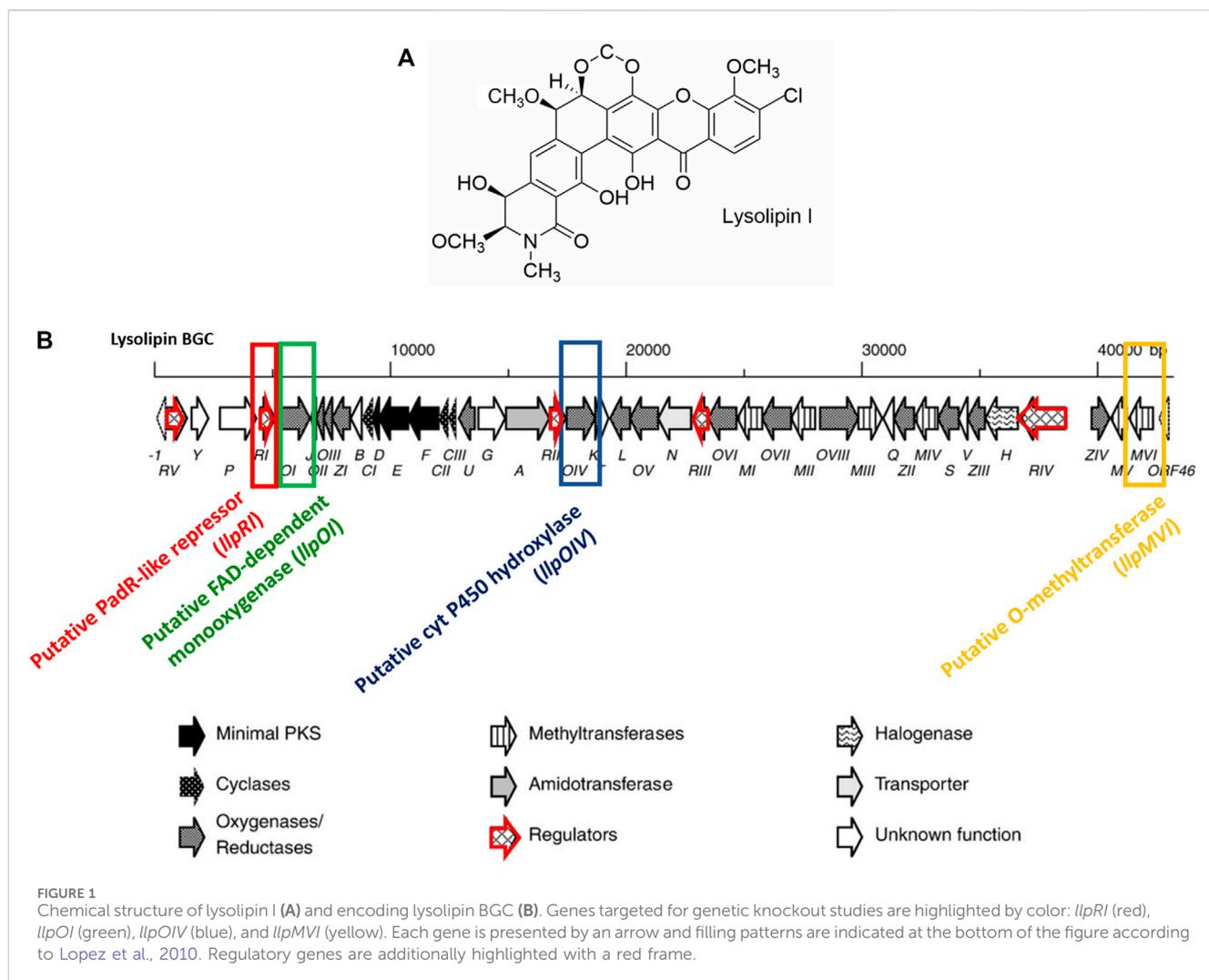
Lysolipin I is a halogenated, polycyclic xanthone natural product belonging to the polyketide class of antibiotics, naturally produced by *Streptomyces violaceoniger* TÜ96 and *Streptomyces tendae* Tü4042. The biosynthesis is encoded on a 43 kb-spanning biosynthetic gene cluster. Heterologous expression of the gene cluster has been established in previous work by using the cosmid 4H04, which was transferred to *Streptomyces albus*. In the current study, we demonstrate the optimization of production yields of therapeutically interesting lysolipin derivatives with extended activity against Gram-negatives and less cytotoxic bioactivities, respectively, by using mutated heterologous *S. albus* producer strains. Production yields were significantly increased by adapting cultivation conditions as well as by inactivating the transcriptional repressor gene *llpRI*, which lead to increased and consistent lysolipin (derivatives) production. Furthermore, cultivation of a *S. albus* 4H04 Δ llpOI mutant strain in bromide-containing fermentation medium resulted in the production of a new brominated lysolipin derivative (C₂₈H₂₀BrNO₉).

KEYWORDS

actinomycetes, *Streptomyces*, antibiotic, polyketides, lysolipin, genetic engineering

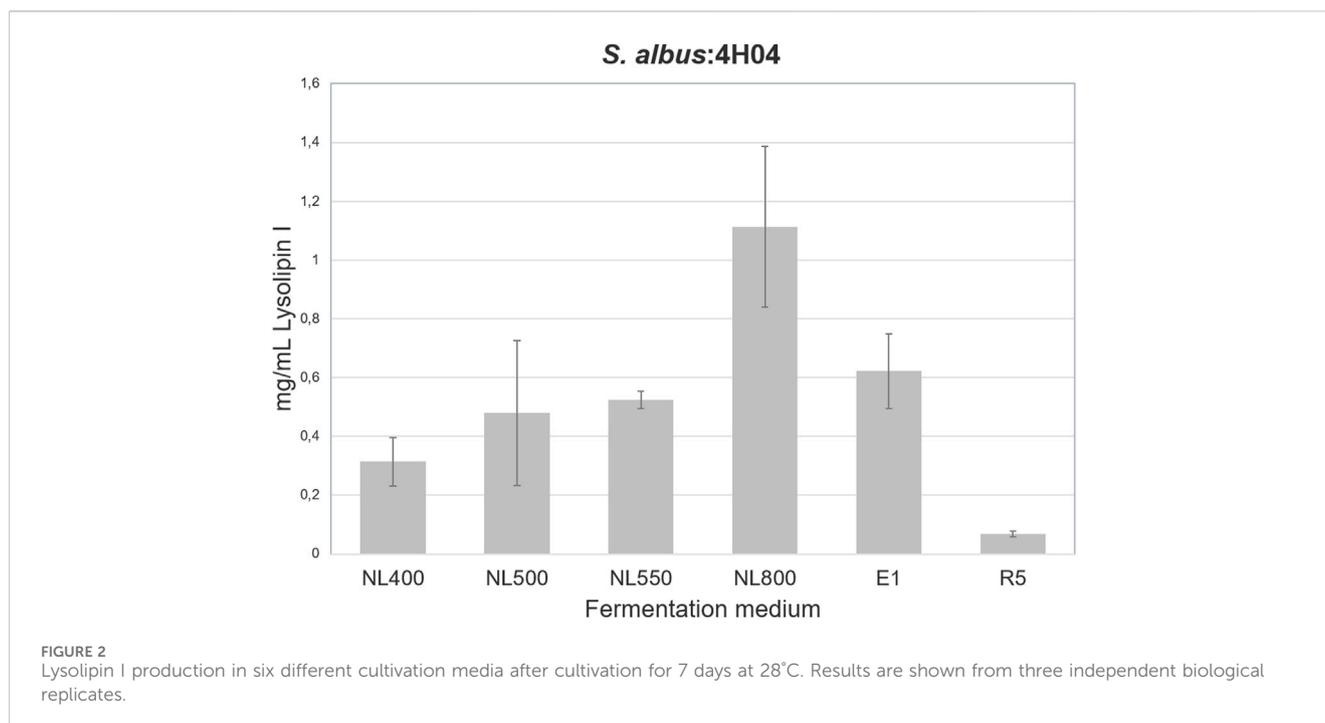
Introduction

Lysolipin I is a polycyclic xanthone natural product belonging to the polyketide class of antibiotics (Figure 1A), which is naturally produced by *Streptomyces violaceoniger* Tü96 and *Streptomyces tendae* Tü4042 (Drautz et al., 1975a; Lopez et al., 2010). It shows an extraordinarily potent antibacterial activity, with a minimum inhibitory concentration (MIC) in the low nanomolar range (less than 10 nM) against Gram-negative microorganisms such as *Proteus* or *Pseudomonas*, as well as Gram-positive microorganisms like *Bacillus*, *Staphylococcus*, *Corynebacterium* (Drautz et al., 1975a). Own recent data show also low nanomolar activity against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) as well as anaerobe microorganisms such as *Helicobacter pylori*. Interestingly, the Gram-negative *E. coli* and *Salmonella typhimurium* were resistant if their cell wall was intact, but susceptible in case of permeation-enhanced strains (Drautz et al., 1975a). The mechanisms of action of the lysolipins bactericidal activity has not been fully elucidated yet, although the compounds are suspected to target the cell envelope, possibly interfering with peptidoglycan synthesis by binding to lipid-bound murein precursors (Drautz et al.,



1975a; Drautz et al., 1975b). The usability of lysolipin as antibacterial agent is limited due to the fact that it also shows cytotoxicity (Lopez et al., 2010). First experiments have shown that structural modifications of the lysolipin core structure can differentially influence antibacterial activity and cytotoxicity (Wohlleben et al., 2005; WO/2006/032232). Modification of compounds can in principle be achieved either by genetic means or by genetic modification of the producer strains. A total chemical synthesis of lysolipin has not been established so far, however, a modular chemical synthesis strategy towards the production of halogenated xanone molecules has been reported recently (Meringdal et al., 2022), which can now serve as a basis for further compound modification. Compound derivatisation of lysolipin by genetic engineering has previously been reported (Wohlleben et al., 2005; WO/2006/032232). Prerequisite for compound derivatisation by genetic engineering is the knowledge of the biosynthetic pathway and the corresponding genes of the producer strains, which has been described for lysolipin biosynthesis by Lopez et al., 2010. However, since production yields of lysolipin were low in the natural producer strains, the biosynthetic gene cluster (BGC), consisting of 44 genes spanning a region of ~43 kb (Figure 1B) was heterologously expressed in *Streptomyces albus* J1074 as expression host, yielding

a stable production of 50 mg/L lysolipin under non-optimized conditions (Wohlleben et al., 2005; WO/2006/032232). Another possibility to increase production yields is genetic manipulation of cluster-situated transcriptional regulatory genes. In the lysolipin BGC, five genes (*llpR1-llpRV*) are present, which code for different types of transcriptional regulators (Lopez et al., 2010). *LlpRI* resembles a transcriptional regulator of the PadR type, which acts as a transcriptional repressor (Rohrer, 2017). *LlpRII* and *LlpRIII* show sequence similarities to transcriptional activators of the TenA family. *LlpRIV* has strong similarity to *Streptomyces* antibiotic regulatory proteins (SARPs), known to act as activators of antibiotic biosynthesis (Rohrer, 2017), whereas *LlpRV* shows sequence similarity to DNA binding proteins from *Streptomyces coelicolor* A3 (2) and contains a helix-turn-helix motif (Rohrer, 2017). The involvement of all individual *llpR* regulatory genes has been demonstrated by mutagenesis in previous work, where deletion of *llpRI* in *S. tendae* Tü 4042 led to significantly increased lysolipin production (>300%; ~0.5 g/L) (Rohrer, 2017) (Supplementary Figure S1). In addition, two genes have been suggested to be involved in conferring resistance against lysolipin, which are *llpN* encoding a potential efflux protein and *llpG* encoding a potential glycosyl transferase (Lopez et al., 2010). Heterologous expression of



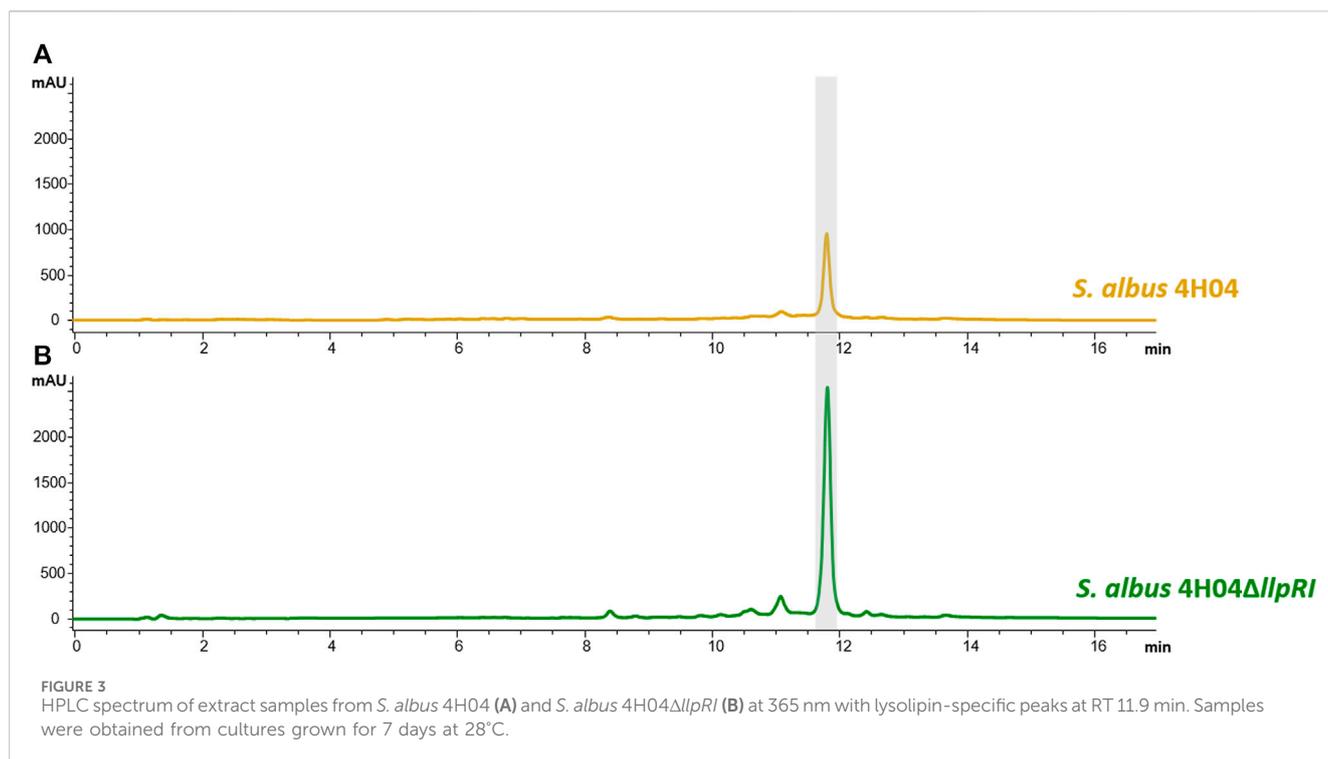
llpN in the lysolipin-sensitive *S. albus* strains led to significantly higher tolerance against lysolipin (up to 500 µg/mL lysolipin) (Unsin, 2008), whereas involvement of *llpG* in conferring resistance to lysolipin has not been demonstrated experimentally. Furthermore, lysolipin was derivatized applying genetic engineering on genes encoding tailoring enzymes of lysolipin biosynthesis. A special structural feature of lysolipin is a xanthone moiety, which is highly decorated by post-polyketide synthase enzymatic reactions catalyzed e.g. by oxygenases, methyltransferases, hydroxylases, a halogenase etc. encoded by the respective modification genes located on the lysolipin BGC (Lopez et al., 2010; Hofeditz et al., 2018). In previous work it has been shown that the deletion of *llpOI* encoding a putative FAD-dependent monooxygenase resulted in the production of a lysolipin derivative CBS40 with improved antibacterial activity against *Escherichia coli*, whereas deletion of *llpOIV*, encoding a putative cytochrome P450 hydroxylase, and *llpMVI*, encoding a putative O-methyltransferase led to lysolipin derivatives CBS68 and a mixture of CBS70 and CBS72, respectively, with less cytotoxicity values (patent WO/2007/079715, Combinature Biopharm, Berlin, Germany). Thus, first experiments have proven that structural modifications of the lysolipin core structure is feasible and can differentially influence antibacterial activity and cytotoxicity.

In the current study, we aimed to optimize lysolipin production yields in the heterologous expression host *S. albus* and generate new lysolipin derivatives based on the already known lysolipin lead structures.

Results and discussion

To define the optimal conditions for lysolipin production, the heterologous host strain *S. albus* 4H04 (Wohlleben et al., 2005;

patent WO/2006/032232) expressing the lysolipin BGC was grown in 14 different cultivation media. For this purpose, *S. albus* 4H04 was cultivated in 100 mL of R5 medium (Handayani et al., 2021) with 100 µg/mL apramycin in a 500-mL Erlenmeyer flasks (with steel springs) on an orbital shaker (180 rpm) at 28°C as a preculture. After 3 days of cultivation, 10 mL of preculture were transferred to 100 mL main culture medium NL200, NL300, NL330, NL400, NL500, NL550, NL800, SGG, TDG, ISP2, ISP3, MS, R5 (Handayani et al., 2021; Wex et al., 2021), or E1 (Hardter et al., 2012), respectively. Cell cultures were grown for 7 days and extracted as reported previously by adjusting the pH to 3, followed by extraction with ethyl acetate (1:1), concentrating samples *in vacuo*, and then re-dissolving in methanol (Lopez et al., 2010). The methanolic extracts were analyzed for lysolipin production by HPLC-DAD-MS analysis as reported previously using a calibration curve with pure lysolipin I as a standard (Lopez et al., 2010). Thereby it was found that NL800 and E1 (Supplementary Table S1) were the best lysolipin production media, yielding 1.1 mg/mL and 0.6 mg/mL of substance, respectively (Figure 2; only six best production media are shown). Regarding the NL800-derived sample, this was >60% more lysolipin in comparison to the previously reported maximal lysolipin production of ~0.5 g/L of the genetically manipulated native lysolipin *S. tendae* producer strain. However, due to the presence of 10 g/L glycerol in NL800, which was detrimental for subsequent chemical analysis and purification of the compound, E1 was selected as suitable production medium for lysolipin in the further work steps. To further increase lysolipin production, the transcriptional repressor gene *llpRI* was targeted for gene inactivation in the *S. albus* 4H04 strain. The *llpRI* mutant was constructed with the REDirect cloning strategy (Gust et al., 2003) as described by Zhang et al., 2018 by using primers listed in Supplementary Table S2. Thereby a *llpRI* disruption cassette with a chloramphenicol (*cam*) resistance marker and artificial MunI restriction sites was introduced into *E. coli* BW25113:



pKD20 harboring the 4H04 cosmid (Supplementary Figure S2). The *llpRI* gene of cosmid 4H04 was deleted via λ -RED-driven recombination yielding 4H04 with an integrated *llpRI* disruption cassette (4H04ΔllpRIcam). The 4H04ΔllpRIcam construct was isolated from *E. coli* and digested with *MunI*. The resulting large (~40 kb) fragment was purified from an agarose gel and religated to yield construct 4H04ΔllpRI. The 4H04ΔllpRI construct was introduced into *S. albus* J1074 by conjugation with *E. coli* ET12567/pUB307 (Flett et al., 2006) as described in Kieser et al., 2000. This resulted in the *llpRI* knockout strain *S. albus* 4H04ΔllpRI. HPLC-MS analyses with extract samples of *S. albus* 4H04ΔllpRI showed that lysolipin I production (peak with retention time (RT) ~11.9 min) is increased 3-fold in comparison to the reference sample *S. albus* 4H04 (Figure 3 A vs. B), proving that LlpRI is acting as transcriptional repressor of lysolipin biosynthesis. A PadR-like DNA binding motif ATGT-8N-ACAT (Kaval et al., 2015) was identified in the intergenic region between *llpRY* and *llpP*, which indicates the binding site for LlpRI. The consistent and strong lysolipin production set the basis to genetically engineer the heterologous host strain to produce therapeutically promising lysolipin derivatives. For that, we specifically focused on optimizing the production of the lysolipin derivatives CBS40, CBS68, and CBS70+72 due to their interesting bioactive properties as outlined in the introduction (Supplementary Table S3). For this purpose, the genes *llpOI*, *llpOIV*, and *llpMVI* were knocked out individually, as the respective mutant strains were expected to produce CBS40, CBS68, and CBS70+72 as lysolipin derivatives, respectively. As the original in-frame deletion strains were no longer available, these mutants were newly constructed exactly as described in patent WO/2007/079715. Thereby, strain *S. albus* 4H04ΔllpOI (\cong CB3919LlpOI_A) contained a mutation of gene *llpOI* encoding the FAD-dependent monooxygenase gene LlpOI, *S. albus* 4H04ΔllpOIV (\cong CB3919LlpOIV) harbored a mutation of gene *llpOIV* encoding the cytochrome P450 hydroxylase

LlpOIV, whereas strain *S. albus* 4H04ΔllpMVI (\cong CB3919LlpMVI_A) contained a mutation of gene *llpMVI*, encoding the methyltransferase LlpMVI. HPLC-MS analysis was carried out with extract samples of the mutant cultures in comparison to extracts from the lysolipin producing *S. albus* 4H04 reference strain. In the CBS terminology lysolipin I is designated as CBS42 with a sum formula of $C_{29}H_{24}ClNO_{11}$ (patent WO/2007/079715). HPLC-MS analysis of extract samples from *S. albus* 4H04ΔllpOI revealed a peak with a RT of ~12.4 min and a mass of $m/z = 550 [M+H]^+$, which correlates to the compound features of CBS40 ($C_{28}H_{20}ClNO_9$) (Figure 4A) (for comparisons see patent WO/2007/079715). Samples from *S. albus* 4H04ΔllpOIV showed a peak at RT 12.1 min and a mass of $m/z = 522.08 [M+H]^+$, correlating to the compound CBS68 ($C_{27}H_{20}ClNO_8$) (Figure 4B), respectively, whereas samples from *S. albus* 4H04ΔllpMVI revealed a specific peak at RT 10.6 min for which two prominent masses were identified at $m/z = 508 [M+H]^+$ and $m/z = 538 [M+H]^+$, which match with the lysolipin derivatives CBS70 ($C_{26}H_{18}ClNO_8$) and CBS72 ($C_{27}H_{20}ClNO_9$), respectively (Figure 4C) (for comparisons see patent WO/2007/079715). Thus, the production of the respective lysolipin derivatives was confirmed.

To optimize production yields of the bioactively interesting lysolipin derivatives, we further modified the strains by genetic engineering attempts and additionally inactivated the *llpRI* gene in those mutants. Here, we focused on the *S. albus* 4H04ΔllpOI and *S. albus* 4H04ΔllpMVI mutant strains, which each produced a broad-spectrum lysolipin derivative (CBS40) or lysolipin derivatives with reduced cytotoxicity (CBS70 and CBS72), respectively (Supplementary Table S3). Mutant construction was performed by the REDirect cloning strategy as reported above by using the pGUS/ΔllpRI deletion construct, which was each introduced into *S. albus* 4H04ΔllpOI and *S. albus* 4H04ΔllpMVI by conjugation with *E. coli* ET12567/pUZ8002, resulting in the strains *S. albus*

been described before as a product from *S. albus* 4H04 Δ llpOI (patent WO/2007/079715), as well as a new brominated version of CBS40 (RT 12.5 min, of $m/z = 594$ [M+H]⁺) with a sum formula of C₂₈H₂₀BrNO₉ (Supplementary Figure S5B).

In this way, numerous lysolipin derivatives could be produced by genetic engineering of the *S. albus* 4H04 heterologous expression strain, whereby lysolipin derivative production was significantly increased by inactivating the repressor gene *llpRI* and a new halogenated lysolipin derivative could be obtained due to the cultivation of the *S. albus* 4H04 Δ llpOI strain in bromide-containing fermentation medium. Altogether, these data hold potential for future drug development attempts with the lysolipin compound.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

HR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–review and editing. SR: Formal Analysis, Investigation, Visualization, Writing–review and editing. AK: Formal Analysis, Methodology, Writing–review and editing. WW: Funding acquisition, Project administration, Resources, Writing–review and editing. YM: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing–original draft, Writing–review and editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchbi.2024.1445095/full#supplementary-material>