Volatile compounds from Actinobacteria as mediators of microbial interactions

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CHAPTER 4

Escherichia coli mediates resistance to volatiles from *Streptomyces* in an OmpR-dependent manner.

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ABSTRACT

The rise of antibiotic resistance in pathogens is a worldwide health problem. *Escherichia coli* is a common bacterium found in human gut and as other bacteria that can rapidly acquire antibiotic resistance and become a life threat. Previously, a chemically mutagenized *Escherichia coli* strain AS19 was isolated based on its enhanced sensitivity to different antibiotics, in particular to actinomycin. The strain was later mutated to study how rRNA modifications confer antibiotic resistance. In this work we use the modified strain *E. coli* AS19-RlmA$^-$ to study the antibiotic effect of *Streptomyces* volatile compounds (VCs). Spontaneous ammonia-resistant *E. coli* derivatives had mutations in the porin master regulator OmpR, resulting in reduced membrane permeability for VCs and enhanced survival. Here we present the genomic and transcriptomic differences of the variant *E. coli* AS19-RlmA$^-$ in response to *Streptomyces* VCs.

INTRODUCTION

Escherichia coli is a Gram-negative bacterium commonly found in the human intestine as well as in other animals (Gorbach 1996). It has an outer membrane that acts as a barrier which limits the number of antibiotics that are effective (Zgurskaya et al 2015). Like other bacteria, *E. coli* pathogenic strains can rapidly acquire antibiotic resistance and become a life threat (Collignon 2009). The emergence of antibiotic resistance is a worldwide problem that reduces or inhibits the efficacy of antibiotics, putting the life of millions of people at risk. Obtaining insights into the genetics of antibiotic resistance is therefore of utmost importance.

E. coli strain AS19 is an actinomycin-sensitive strain that was selected to study antibiotic sensitivity and how this might be linked to cell permeability (Avalos et al 2018a). The strain was obtained by chemical mutagenesis of *E. coli* strain B with N-methyl-N'-nitroso-N-nitroguanidine (Sekiguchi and Iida 1967). AS19 has been used further in studies of

bacteriophage infection (Iida and Sekiguchi 1971) and antibiotic resistance/sensitivity (Liu and Douthwaite 2002b). In a more recent study, the derivative *E. coli* AS19-RlmA$^-$ (referred to as *E. coli* ASD19 from now on) was developed to examine how rRNA modifications affect susceptibility to MLS_B and ketolide antibiotics (Liu and Douthwaite 2002a). This latter strain harbors a kanamycin resistance cassette disrupting the rRNA methyltransferase gene *rlmA* (formerly *rrmA*).

The fact that *E. coli* AS19 has a higher permeability towards actinomycin (Sekiguchi and Iida 1967) as well as a general higher sensitivity towards a majority of antibiotics appeals to its use as a test microorganism for antibiotics that might be present in lower concentrations. The gaseous nature of volatile compounds (VCs) makes the accumulation of such molecules difficult but allows them to disperse over longer distances or areas. This trait enables these compounds to act as infochemicals in long distance communication. The sensitivity of *E. coli* ASD19 towards antimicrobial VCs (AMVCs) as shown in Chapter 3, will serve as a starting point to study how bacteria respond and protect themselves against these molecules.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Streptomyces sp. MBT11 (Zhu et al 2014) and *Streptomyces venezuelae* (Song et al 2016) were used as antimicrobial volatile producers. *E. coli* BREL606 (Daegelen et al 2009) and its hypersusceptible variant *E. coli* AS19-RlmA$^-$ (Liu and Douthwaite 2002a) referred to as *E. coli* ASD19 from now on were used as test strains. Volatile antimicrobial assays were performed using a petri dish with two compartments, one filled with SFM agar (Soya Flour Mannitol) for *Streptomyces* growth (Kieser et al 2000) and the other half with LB agar for *E. coli* growth (Sambrook 1989). *Streptomyces* sp. MBT11 and *S. venezuelae* were streaked on the SFM half and allowed to grow for 5 days at 30°C. After that, *E. coli* strains were inoculated on the LB side using a concentration of 10^4 cfu/mL. A resistant

E. coli ASD19 mutant (ARM9) insensitive to antimicrobial volatile compounds (AMVCs) was obtained spontaneously after prolonged growth under *Streptomyces* sp. MBT11 VCs.

Minimal inhibitory concentration (MIC)

Two-fold serial dilutions were made with LB in 96-well plates. Wells were inoculated in triplicates with a final concentration of 10^5 cfu/mL (3µL of a dilution made from a fresh culture grown to an O.D. = 1.0 and diluted 100 times to give a concentration of 10^7 cfu/mL (100 µL culture O.D. = 1 + 9.9 mL LB)). The final volume in each well was 150 µL. Plates were incubated overnight at 37°C and OD_{600} was measured after 20 h of growth. The MIC was assigned as the lowest concentration where the O.D was not higher than the LB not inoculated.

Whole genome sequencing

Genomic DNA from *E. coli* was isolated as described elsewhere (Sambrook 1989). Briefly, *E. coli* cells were harvested from an overnight culture and re-suspended in lysis buffer (TE, SDS 10%, Proteinase K), incubated for 1h at 37°C. classical extraction with phenol-chloroform was performed and the aqueous layer was precipitated with absolute ethanol. The DNA pellet was washed with 70% Ethanol, dried and solubilized in TE to perform RNA digestion with RNase 50 µg/mL (RNase A, Thermo Fischer). Degraded RNA was removed by phenol/chloroform extraction followed by ethanol precipitation. DNA was re-suspended in nuclease-free water.

Genome sequencing of *E. coli* ASD19 and its mutant ARM9 was performed using Illumina HiSEQ and PacBio RS at Baseclear BV, Leiden (The Netherlands). Paired-end sequence reads were generated using the Illumina HiSeq 2000 system and mapping the individual reads against the reference genome of *E. coli* B str. REL606. The contigs were placed into superscaffolds based on the alignment of the PacBio CLC reads. Alignment

was performed with BLASR (Chaisson and Tesler 2012). Genome annotation was performed using the Baseclear annotation pipeline based on the Prokaryotic Genome Annotation System (http://vicbioinformatics.com). Variant detection was performed using the CLC genomics workbench version 6.5 (QIAGEN Bioinformatics). The initial list of variants was filtered using the Phred quality score and false positives were reduced by setting the minimum variant frequency to 70% and the minimum number of reads that should cover a position was set to 10. Relevant mutations were confirmed by PCR analysis. The genome has been submitted to the NCBI; the accession number is CP027430.

RNA sequencing

For RNA extraction, *E. coli* cells were grown to an OD_{600} of 0.5, RNA Protect Bacteria Reagent (Qiagen Cat No. 76506) was added according to manufacturer instructions. Cells were pelleted and re-suspended in boiling 2% SDS + 16mM EDTA followed by extraction with Phenol:chloroform:Isoamyl alcohol (25:24:1) pH 6.6. (VWR Prolabo 436734C). Aqueous phase was precipitated with 3M sodium acetate pH 5.2 and pure ethanol, washed with 70% ethanol and re-suspended in RNAse free water. DNA was removed using 5 units of DNAse I (Fischer Scientific, The Netherlands) with further purification using again phenol:chloroform:isoamyl alcohol and precipitation with sodium acetate and ethanol. The final pellet was dissolved in RNase free water.

RNA sequencing and analysis was performed by Baseclear BV (Leiden, The Netherlands). For this, the RNA quality was determined using a Bioanalyzer. Ribosomal RNA was subsequently removed with a Ribo-Zero kit (Epicenter) and the remaining RNA used as input for the Illumina TruSeq RNA-seq library preparation. Once fragmented, it was converted into double strand cDNA, the fragments (on average 100-200 bp) were ligated with DNA adapters at both ends and amplified via PCR. The resulting library was then sequenced using Illumina Sequencing. The FASTQ sequence reads were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessment was based on data passing the

Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signals were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.10.0.

For the RNA-Seq analysis the quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the "Trim sequences" option present in CLC Genomics Workbench Version 6.0.4. The quality-filtered sequence reads were used for further analysis with CLC Genomics Workbench. First an alignment against the reference and calculation of the transcript levels was performed using the "RNA-Seq" option. Subsequent comparison of transcript levels between strains and statistical analysis was done with the "Expression analysis" option, calculating so-called RPKM values. These are defined as the Reads Per Kilobase per Million mapped reads (Mortazavi et al 2008) and seeks to normalize for the difference in number of mapped reads between samples as well as the transcript length. It is given by dividing the total number of exon reads by the number of mapped reads (in Millions) times the exon length (in kilobases).

The RNAseq data has been submitted to the GEO (Gene Expression Omnibus) from NCBI (National Biotechnology Center Information). The GEO accession number is GSE111370.

Volatile suppressor mutant. *ompR* and *envZ* complementation.

E. coli strain ASD19 suppressor mutant ARM9 was complemented by inserting the *ompR* or *envZ* genes in pCA24N from the ASKA collection (Kitagawa et al 2005). Cells of suppressor mutant ARM9 containing the plasmid were inoculated in LB + chloramphenicol (25µg/mL) with or without IPTG 0.1 mM for induction of the gene expression.

RESULTS

Escherichia coli ASD19 sensitive to soluble and volatile antibiotics

Escherichia coli strain AS19 is an actinomycin-sensitive strain that is also sensitive to other antibiotics like tylosin, erythromycin and streptomycin (Table 1).

Table 1. Minimum inhibitory concentrations (MIC, μg/mL) of *E. coli* Brel606 and *E. coli* ASD19.

	Brel606	**ASD19**
Antibiotic	μg/mL	
Ampicillin	3.9	1
Erythromycin	80	1.25
Kanamycin	8	>500
Polymyxin	0.06	0.25
Spectinomycin	31.25	31.25
Streptomycin	>500	4
Tetracycline	<1	<1
Tylosin	500	15.6

Both *E. coli* strains were tested for its sensitivity against antibacterial volatile compounds released by streptomycetes. *Escherichia coli* ASD19 was chosen as the indicator strain. As shown in Chapter 3, growth of these strains is completely inhibited when exposed to VCs from *Streptomyces* sp. MBT11.

E. coli resistance to volatile ammonia from *Streptomyces* sp. MBT11

After 48 h of incubation adjacent to *Streptomyces* MBT11, a few colonies appeared, that had likely undergone suppressor mutations. These colonies were streaked again to test if they had indeed become resistant to the AMVCs produced by *Streptomyces* sp. MBT11. Four colonies that showed different levels of resistance as indicated by the number of colonies and its size were further analyzed (Figure 1A). Of these,

suppressor mutant ARM9 was selected for its higher resistance (Figure 1A, far right). As shown in Chapter 3, *Streptomyces* sp. MBT11 produces high concentrations of ammonia. It is logical to assume that ammonia is the main AMVC and that ARM9 had sustained one or more suppressor mutations to enable ammonia resistance. This notion is supported by the fact that ARM9 was also resistant against AMVCs from *S. venezuelae* (Figure 1B), which is also known to produce high concentrations of ammonia.

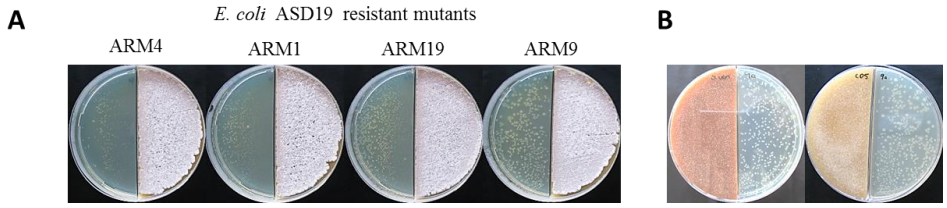

Figure 1. Colonies from *E. coli* strain ASD19 with different resistance patterns to VCs from *Streptomyces* sp. MBT11. **B.** Strain ARM9 is completely resistant to VCs from *S. venezuelae* (left) and *Streptomyces* sp. MBT11.

Strain ASD19 and ARM9 were grown under different concentrations of ammonia. The suppressor mutant ARM9 was more resistant to ammonia than its parent strain, with MICs of 25 mM and 20 mM, respectively (Figure 2). This is a small but very significant difference, as we previously showed that 20 mM is the tipping point between ammonia resistance and sensitivity.

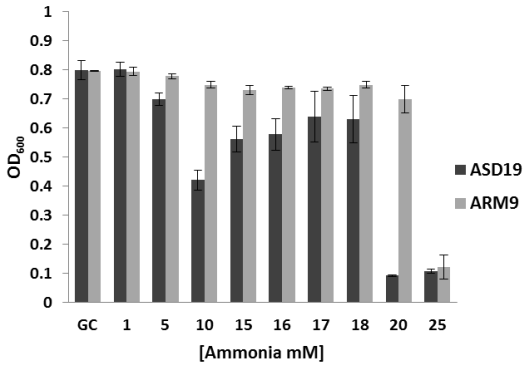

Figure 2. Growth of *E. coli* strain ASD19 (black) and *E. coli* strain ASD19 suppressor mutant ARM9 (gray) under the presence of different concentrations of ammonia.

OmpR is key to ammonia toxicity

It is known that under low availability of nitrogen, the AmtB transporter facilitates the intake of ammonium inside the cell (Conroy et al 2007, Wirén and Merrick 2004). Our conditions include high concentrations of ammonia; therefore we hypothesised that a mechanism other than the AmtB channel would be involved in the resistance towards ammonia. To identify the nature of the mutation(s) sustained by ARM9, its genome sequence was compared to that of its parent *E. coli* ASD19 (Supplementary Table S1). In total 658 mutations were found by SNP analysis, of which 198 gave rise to amino acid changes, insertions or deletions. However, one change immediately stood out, namely the introduction of two insertion elements (insA_31 and insB_31) in-between the -35 and -10 consensus sequences of the promoter for *ompR*-envZ, which encode the two-component system (TCS) consisting of response regulator OmpR and sensory kinase EnvZ (Figure 3). This TCS is involved in osmoregulation in response to environmental signals (Nikaido 2003) and regulates the expression of outer membrane porins OmpF and OmpC. Importantly, these are known to be involved in antibiotic resistance regulated by osmotic pressure and pH (Fernandez and Hancock 2012), and to reduce the responsiveness of *E. coli* cells to VCs (Kim et al 2013).

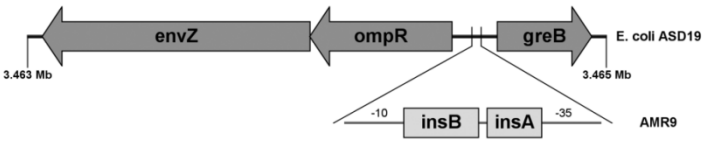

Figure 3. Diagram of the insertion sequences in the middle of the promotor region for *ompR/envZ* genes.

Reduced transcription of the *ompR-envZ* operon is the cause of ammonia resistance

Considering the location right in the middle of the promoter, we expected that the insertion elements (IS) in the *ompR-envZ* promoter reduced the transcription of these crucial TCS genes. To establish the transcriptional consequences of the IS insertion into the *ompR-envZ* promoter region, RNAseq was performed on *E. coli* ASD19 and its suppressor mutant ARM9 grown in LB media until mid-exponential phase (OD_{600} 0.5), and the global transcription profiles compared (see Supplementary Table S2 for the full dataset). Table 2 shows genes highly up/down regulated as a result of a clustering analysis using a cut-off value of a fold change +/- 2.0. These data confirm the downregulation of *ompR* and *envZ* genes and other related genetic elements like *omrA*, a small mRNA that negatively regulates *ompR* expression. Additionally, genes involved in amino-acid metabolism were down regulated, including the *astABCE* gene cluster involved in the ammonia-producing arginine catabolic pathway, *aspA* that is involved in the conversion of L-aspartate into fumarate and ammonia, and *tnaC* for catabolism of tryptophan, which again releases ammonia. Furthermore, the toxin-antitoxin system GhoT-GhoS (Wang et al 2012) appeared up-regulated. These toxin-antitoxin systems are known to be involved in the formation of persister cells (tolerant to antibiotics without undergoing genetic change) (Dorr et al 2010, Kim and Wood 2010). We lack evidence that this toxin-antitoxin system participates in the resistance to AMVCs, however we also observe down regulation of tRNAs which is linked to bacterial persistence by arrest of transcription (Que et al 2013).

Table 2. Clustering of the differentially down/up-regulated genes in *E. coli* ARM9 compared to *E. coli* ASD19.

Cluster	Differentially expressed genes	Function	Fold Change
		DOWN-REGULATED	
Membrane Function/ Transport	*envZ*	sensory histidine kinase in two-component regulatory system with OmpR	-16.43
	omrA	small regulatory RNA	-15.86
	yhdV	putative outer membrane protein	-15.86
	ompR	response regulator in two-component regulatory system with EnvZ	-14.84
	dacD	D-alanyl-D-alanine carboxypeptidase, penicillin-binding protein 6b	-6.47
	yqhH	outer membrane lipoprotein, Lpp paralog	-6.20
	ydiM	putative MFS transporter, membrane protein	-4.83
	yiaD	multicopy suppressor of bamB, outer membrane lipoprotein	-4.54
	yajR	putative transporter	-4.37
	yhfL	small lipoprotein	-3.22
Domain: EAL	*bluF*	anti-repressor for YcgE, blue light-responsive, FAD-binding, inactive c-di-GMP phosphodiesterase-like EAL domain protein	-2.55
	yhjH	cyclic-di-GMP phosphodiesterase, FlhDC-regulated	-2.13

	ycgG	putative membrane-anchored cyclic-di-GMP phosphodiesterase	-2.36
	yliE	putative membrane-anchored cyclic-di-GMP phosphodiesterase	-2.04
Aminoacyl-tRNA biosynthesis	*alaW*	tRNA-Ala	-2.02
	alaX	tRNA-Ala	-2.30
	argX	tRNA-Arg	-2.19
	asnV	tRNA-Asn	-2.76
	asnW	tRNA-Asn	-2.12
	glnV	tRNA-Gln	-2.18
	glnX	tRNA-Gln	-2.35
	hisR	tRNA-His	-2.03
	leuP	tRNA-Leu	-2.37
	leuT	tRNA-Leu	-3.34
	leuU	tRNA-Leu	-2.43
	pheV	tRNA-Phe	-3.56
	proK	tRNA-Pro	-2.24
	proL	tRNA-Pro	-2.94
	proM	tRNA-Pro	-2.76
	selC	tRNA-Sec	-2.07
	valV	tRNA-Val	-3.52
Aminoacid	*astA*	arginine succinyltransferase	-2.17

metabolism	*astB*	succinylarginine dihydrolase	-2.09
	astC	succinylornithine transaminase, PLP-dependent	-2.13
	astE	succinylglutamate desuccinylase	-2.14
	feaR	transcriptional activator for *tynA* and *feaB*	-2.02
	tnaC	tryptophanase leader peptide	-2.21
Fatty acid Oxidation	*fadA*	3-ketoacyl-CoA thiolase (thiolase I)	-2.20
	fadB	fatty acid oxidation complex, α component	-2.19
	fadH	2,4-dienoyl-CoA reductase, NADH and FMN-linked	-2.14
	prpB	2-methylisocitrate lyase	-2.07
		UP-REGULATED	
Prophage	*xisD*	pseudogene, exisionase in defective prophage DLP12	7.252
	ylcI	DUF3950 family protein, DLP12 prophage	5.318
Ribosome	*rrsC*	16S ribosomal RNA	3.93
	rrfB		3.32
	rrfC		2.18
	rrfD	5S ribosomal RNA	5.08
	rrfG		3.55
	rrfH		3.38
Pilus	*fimC*	periplasmic chaperone	2.42
	fimF	minor component of type 1 fimbriae	2.05
	ppdD	putative prepilin peptidase-dependent pilin	4.35
	ydeR	putative fimbrial-like adhesin protein	2.18

To confirm that indeed the reduced transcription of *ompR-envZ* is the major cause for the acquired ammonia resistance, *E. coli* mutant ARM9 was genetically complemented by the introduction of constructs from the ASKA collection (Kitagawa et al 2005) expressing either *ompR* or *envZ*. Introduction of constructs expressing either *ompR* or *envZ* restored ammonia sensitivity, while transformants harboring the empty plasmid continued to be resistant (Figure 4). This strongly suggests that the reduced expression of *ompR* and *envZ* is the main cause of the acquired ammonia resistance.

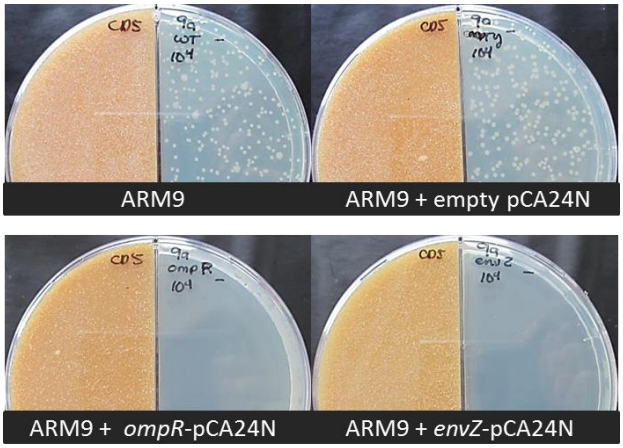

Figure 4. *E. coli* strain ASD19 suppressor mutant ARM9, complemented with empty pCA24N plasmid, *ompR*-pCA24N, *envZ*-pCA24N.

Taken together, these data show that *E. coli* responds to exposure to ammonia by reducing *ompR-envZ* transcription, down regulating the expression of OMPs to minimize the passage of small molecules, and by the reduction of ammonia biosynthesis. Both responses are aimed at defense against the accumulation of toxic levels of ammonia. A similar response was shown previously when an *ompR* mutant did not become as resistant to tetracycline as the wild-type *E. coli* when exposed to ammonia (Bernier et al 2011). Our results show that reducing the expression of OMPs is a defense mechanism against ammonia toxicity extending also earlier observations that *ompF* mutants show impaired response to VOCs that affect the motility of *E. coli* (Kim et al 2013).

DISCUSSION

In Chapter 3, it was shown that *Streptomyces* sp. MBT11 inhibits the growth of *E. coli* strain ASD19 via the production of large amounts of ammonia that accumulate over large distances. In this Chapter, this feature was exploited to study the molecular basis for ammonia resistance, by selecting for spontaneous suppressor mutants of *E. coli* strain ASD19 that have become resistant to volatile antibiosis caused by MBT11. Mutant ARM9 was selected, and genome sequencing revealed the insertion of a duplicated mobile element (insA_31 and insB_31) in the promoter region of the *ompR-envZ* operon. This resulted in the strongly reduced expression of the two-component system *ompR/envZ* that mediates the signal transduction in response to environmental osmolarity changes. This system is known to regulate the major outer membrane porins OmpC and OmpF. In *E. coli,* OmpC and OmpF are known to play a key role in antibiotic resistance (Fernandez and Hancock 2012) thereby reducing the number of entry channels. OmpC and OmpF are two of the main general porins that form a size-defined channel for the diffusion of hydrophilic molecules. The estimated amount of porins in a cell can reach up to 10^6 copies (Achouak et al 2001). These porins are regulated by environmental stimuli such as osmotic pressure and pH, therefore, when the regulator of such proteins is no longer expressed, the amount of porins present in the outer membrane will be much lower reducing the permeability to toxic compounds into the cell, thus increasing the resistance to antimicrobials (Jaffe et al 1982). Previously, an *ompF* mutant showed impaired response to VOCs affecting motility in *E. coli* (Kim et al 2013). Our results show that the suppression of the porin regulator 'OmpR' is responsible for the resistant phenotype, most likely because hydrophilic molecules such as ammonia and other AMVCs can no longer enter the cell in high amounts. Besides this, several studies show that resistance related to changes in the porins may only contribute partially to the overall resistance of a cell. For this reason, it is not uncommon to see that the effect of porin mutations is enhanced by additional mechanisms. For example, some strains of *Serratia marcescens* overproduce the beta-lactamase AmpC and also lack the porin OmpF (Suh et al 2010). *E. coli* clinical isolates lacking both *ompC* and *ompF* also showed an increased production of beta-lactamase (Beceiro et al 2011).

Our results show that the main effect is exerted by the down-regulation of *ompR-envZ*, supported by the loss of the resistance phenotype when the mutant ARM9 is complemented with the regulatory genes *ompR* and *envZ* confirming the idea that a mutant disturbing the *ompB* operon (*ompR*/*envZ*) is no longer affected by high concentrations of ammonia.

The fact that ammonia is a major player in the antimicrobial effect of VCs against *E. coli*, is reinforced by the down-regulation of genes related to amino acid metabolism with a collateral release of ammonia such as the arginine catabolic pathway (*astABCE*); *aspC* which converts L-aspartate into fumarate and ammonia and *tnaC*, the leader peptide of the tryptophanase operon involved in tryptophan degradation releasing ammonia. When cells are exposed to high amounts of toxic molecules that are in fact routine primary metabolites, such as ammonia, a negative feedback loop is a logical survival strategy, with the aim to reduce accumulation of the causing agent.

Ammonia is an important nitrogen source for most bacteria, however, our work shows that it becomes toxic when present in high amounts. Some strains of *Streptomyces* are capable of producing high concentrations of this volatile compound turning it into an antibiotic against bacteria like *E. coli.*

In conclusion, *E. coli* ASD19 is sensitive to high concentrations of ammonia and this work presents the genetic and transcriptomic basis of *E. coli* survival response against toxic concentrations of this volatile. Volatile compounds are implicated in diverse inter and intra-specific interactions all over different ecological niches, from soil to clinical environments. Therefore, the knowledge and understanding of the mechanisms behind such interactions is of great relevance.