Characterization and direct molecular discrimination of *rpsL* mutations leading to high streptomycin resistance in *Erwinia amylovora*

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Abstract

Erwinia amylovora can rapidly become highly resistant to applications of the antibiotic streptomycin through a single nucleotide mutation in the *rpsL* gene, which causes an amino acid substitution that prevents inhibitory binding of streptomycin while preserving the functionality of the ribosome. Several viable mutations are described in the literature, but screenings in the orchards consistently revealed that the one leading to the substitution of lysin by arginine at position 43 (K43R) is by far the most abundant. In this work, we tested the rate of emergence of the various spontaneous mutants, their tolerance to different antibiotic concentrations and their general fitness in competition experiments. Furthermore, we developed a molecular assay based on the SNaPshot™ Multiplex Kit from Thermo Fisher Scientific that allows swift determination of *rpsL* mutations using a single-base primer extension approach directly from colonies on plate. Or results indicate that the prevalence of variant K43R is largely due to fact that it retains the highest environmental fitness even in absence of antibiotic pressure. Sequence analysis of two resistant colonies that did not respond as expected in the SNaPshot™ assay revealed the existence of two yet undescribed *rpsL* mutations resulting in conditional-lethal streptomycin-dependent (Sm^D) phenotypes, which are unable to grow in absence of the antibiotic.

Introduction

In North America, the dependence of pome fruit production on streptomycin has become a mounting problem in recent years due to the emergence of streptomycin-resistant (Sm^R) *Erwinia amylovora* variants (Tancos and Cox 2016). Two mechanisms were identified as the origin of streptomycin resistance in *E. amylovora*. Resistance to moderate concentrations of the antibiotic (up to approximately 500-750 μg ml^{-1}) is known to be conferred by the presence

of aminoglycoside-altering enzymes within the pathogen (McManus et al. 2002). These enzymes modify streptomycin to a nontoxic form and are encoded by the gene pair *strA-strB* (Chiou and Jones 1995; Rezzonico et al. 2009), which is typically found within the transposable element Tn5393 that is found in association either to conjugative or on nonconjugative plasmids (Förster et al. 2015; McGhee and Sundin 2012).

On the other side, the causal agent of fire blight can quickly become resistant to high levels of streptomycin (i.e., exceeding 2'000 μg ml^{-1}) through a single point mutation in the chromosomal *rpsL* gene encoding the S12 protein of the 30S small ribosomal subunit (McManus et al. 2002). This causes an amino acid substitution that prevents inhibitory binding of streptomycin while preserving the functionality of the ribosome. Screening of highly resistant bacteria in the orchards typically yields the same mutation at codon 43 of the *rpsL* gene, which leads to the substitution of a lysine through arginine (K43R) in the S12 protein. Nevertheless, alternative mutations involving the replacement of a lysine, either at the same codon (K43T, K43N) or at another position in the protein (K88R), have also been found to result in Sm^R isolates in the field or under laboratory conditions (Chiou and Jones 1995).

In this work, we address the question whether the prevalence of the K43R variant among the population of Sm^R *E. amylovora* in the orchards is due to a higher probability of random mutational emergence of this particular genotype, to its tolerance to increased antibiotic concentrations or is the result of an improved general fitness with respect to the other variants. For this purpose, the relative frequency of spontaneous appearance of the different *rpsL* mutations was assessed by testing random Sm^R colonies obtained from overnight cultures grown without antibiotic pressure in LB medium. In order to swiftly characterize these mutations, we developed a molecular assay based on the SNaPshot® Multiplex Kit (Thermo Fisher Scientific) that is able to discriminate all the above variants without the need of performing Sanger sequencing. The resistance level to streptomycin of the available genotypes was tested by assessing the minimal inhibitory concentrations (MICs) for the antibiotic *in vitro*. Furthermore, to assess the overall fitness of the different mutants, their growth profiles were tested in various media with or without selective pressure using optical densitometry and plate counts both separately and in competitive assays.

Material and methods

Bacterial strains

The strains used in this study are listed in **Table 1**. They were all own laboratory Sm^R derivatives collected throughout the years of *E. amylovora* CFBP 1376, which was originally obtained from *Collection Française de Bactéries Phytopathogènes* (INRA Angers, France) (Smits et al. 2010). Bacteria were routinely cultivated in Luria Bertani (LB) broth or on LB agar plates (Conda Pronadisa Lab, Madrid, Spain) as well as on LB agar plates amended with 100 µg ml^{-1} of streptomycin (LB + Sm^{100}). In selected experiments, basal medium supplemented with sugars, mimicking the nutrient composition of apple flowers, was also used (modified after (Pusey 1999)) (**Table S1**).

Determination of fitness and minimal inhibitory concentration (MIC)

The strains were pre-cultivated overnight in LB broth without streptomycin at 28°C with continuous shaking (180 rpm), the absorbance was measured and adjusted to an OD_{600} =0.01 on a total volume of 10 ml LB or basal medium. The bacterial suspension was aliquoted into different Eppendorf tubes, one without the addition of the antibiotic and the rest containing varying concentrations of streptomycin (100 to 16'000 µg ml^{-1} according to the experiment). Individual samples were distributed into 100-wells honeycomb sterile plates (200 µl per well) and growth curves were recorded in quintuplicates by measuring the optical density of the bacterial suspensions over time using the Bioscreen C device (Oy Growth Curves Ab Ltd., Helsinki, Finland). Average growth rates were calculated by taking the first and final absorbance of the exponential phase (Smits et al. 2002).

Competition assays

Two co-cultivation experiments lasting four and eight days, respectively, were conducted to compare how each Sm^R mutant is able to compete against the wild-type strain CFBP 1376 (**Figure 1**). The two experiments were essentially identical, with the exception that the first one was terminated after plating the co-cultures at the end of day 4. Each variant was initially inoculated in a 1:1 ratio with the wild-type strain in co-cultures containing the basal medium described above. At least three replicate measurements (i.e., independent starting cultures) were used for each strain combination and their results were averaged. The starting co-culture was prepared in a total volume of 4 ml by adding 500-µl suspensions of wild-type CFBP 1376 and of the selected Sm^R variant, both adjusted to an initial OD_{600} of 0.125 after washing in the same medium. After overnight incubation at 28°C / 180 rpm a volume of 4 µl was transferred to a new tube containing 4 ml of minimal medium (1:1000 dilution) to initiate the first of a series of seven successive 24-hours subcultivation steps. Samples were taken after one, three,

six and eight days and serially diluted in sterile 0.9% NaCl. Total colony forming units (CFUs) and Sm^R CFUs were determined in triplicates by plating appropriate dilutions onto LB agar and LB + Sm^{100} agar, respectively. Plates were incubated at 28°C for 36 h prior to counting. Once the colonies were visible, the number of CFUs was calculated using the following equation:

$$CFU\left(\frac{\#}{ml}\right) = No.of\ colonies * \frac{1}{dilution\ factor} * \frac{1}{sample\ volume}$$

PCR amplification of the rpsL *gene*

The PCR amplification of the whole *rpsL*-gene and its flanking regions was performed using the KAPA2G Robust HotStart ReadyMix PCR kit (Roche) with primer pair E*rpsL*-fw/E*rpsL*-rev (**Table 2**) at a final concentration of 0.5 µM as recommended by the manufacturer's protocol. The length of the obtained amplicon was 487 bp. Boiled cells of *E. amylovora* were used as template, either derived from a 1:20 dilution of an overnight culture or directly picked from a single colony on plate and resuspended in PCR-grade water. PCR conditions consisted in an initial denaturation for 3 min at 95°C, followed by 35 cycles of 15 sec denaturation at 95°C, 15 sec annealing at 60°C and 15 sec elongation at 72°C, and a final elongation of 1 min at 72°C. PCR products were cleaned using MultiScreen PCRµ96 filter plates (Merck Millipore) and used as templates for the SNaPshot® multiplex PCR.

Development of a SNaPshot® assay targeting rpsL *mutants*

In order to detect the different known mutations of the *rpsL* gene leading to streptomycin resistance without having to resort to Sanger sequencing, we developed an assay based on the the SNaPshot® multiplex PCR Kit (Thermo Fisher Scientific). This approach allows interrogating one or multiple SNP positions via a single-base extension reaction of suitable primer(s) followed by analysis in the ABI3500 Genetic Analyzer (Thermo Fisher Scientific) (**Figure 2**).

A sets of three SNaPshot® primers was designed and tested to interrogate the known variable bases in codons 43 (2^{nd} and 3^{rd} base) and 88 (2^{nd} base). Primer sequences were analyzed for complementarity, formation of secondary structures, and specificity. The primers were designed to anneal immediately adjacent to the expected mutation sites either on the forward (primer *rpsL*_43f) or on the reverse strand (primers *rpsL*_43r and *rpsL*_88r). The length of their template-annealing 3'-end was adjusted in order to obtain comparable base-stacking

melting temperatures of 58-60°C (von Ahsen et al. 1999). Each primer was synthesized with a 5'- tail of not-annealing nucleotides to allow separation of SNaPshot® products by capillary electrophoresis on the basis of their size (**Table 2**).

The concentrations of the components of the SNaPshot® multiplex PCR were optimized in order to obtain, for all three primers, signals of approximately the same intensity on the ABI3500 Genetic Analyzer. Furthermore, a reduction in the use if the consumables, and consequently the price of the assay, was achieved by reducing the reaction volume from 10 µl to 5 µl and by reducing the concentration of the SNaPshot® multiplex mix from 1x to 0.25x strength. Final concentrations for the SNaPshot® multiplex PCR are indicated in **Table 3**. SNaPshot® multiplex PCR cycling conditions consisted in 25 cycles of 10 sec denaturation at 96°C, 5 sec annealing at 50°C and 30 sec elongation at 60°C.

Detection of mutations was realized on an ABI3500 Genetic Analyzer. Each completed SNaPshot® PCR was supplemented with 0.5 µl Shrimp Alkaline Phosphatase (New England Biolabs) and incubated for 90 min at 37°C to remove unincorporated ddNTPs. An aliquot of 0.5 µl of the resulting mix was then added, together with 0.5 µl GeneScan™ 600 LIZ™ dye Size Standard v2.0 (Thermo Fisher Scientific), to 9 µl of Hi-Di™ Formamide (Thermo Fisher Scientific). The mixture was denatured for 5 min at 95°C in a thermal cycler and snap-cooled on ice before being loaded into a 96-well plate and analyzed on the ABI3500 Genetic Analyzer.

Estimation of the relative appearance rate of spontaneous rpsL *mutations*

The relative *in vitro* frequency of spontaneous appearance of the different *rpsL* mutations was assessed by testing random colonies obtained from overnight cultures at 28°C grown without antibiotic pressure in LB medium. Centrifugation-concentrated aliquots of the cultures were spread on LB + Sm^{100} Agar after which emerging colonies were sub-cultivated in 200-µl LB + Sm^{100} broth microcultures. Cells were boiled and diluted 1:20 in PCR-grade water as described above, then tested for their genotype using the developed SNaPshot® Multiplex protocol.

Sanger sequencing

Colonies, yielding ambiguous results in the SNaPshot® Multiplex, were reanalyzed by Sanger sequencing of the PCR amplicon, obtained at the beginning of the procedure with the same primers E*rpsL*-fw and E*rpsL*-rev used for its amplification. The BigDye™ Terminator v3.1

Matrix Standard Kit (Thermo Fisher Scientific) was used, using 5-10 ng of the purified PCR product as input template, to perform the sequencing reaction according to the manufacturers' instruction.

Results

Determination of minimal inhibitory concentration (MIC)

The determination of the minimal inhibitory concentration (MIC) was performed to determine whether the various Sm^R mutants displayed different tolerance levels in terms of maximum survivable streptomycin concentrations under different growing conditions, i.e. either in LB broth or in minimal medium. With the exception of wild-type CFPB 1376 that was inhibited already by minimal concentrations (< 20 μg ml^{-1}), most variants were able to grow in rich medium (LB) containing streptomycin up to 16'000 μg ml^{-1} (**Figure S1**) at replication rates that were not dissimilar to that of the wild-type in the same medium without antibiotic (**Table S2**). The only exception was variant K88R that, with respect to the K43 mutants, generally displayed a perceptibly slower growth rate. Furthermore, the growth rate of variant K88R shows a noticeable decrease with increasing streptomycin concentrations that is not obvious in other variants. In LB medium without streptomycin, the different variants generally displayed a greater variability in their growth behavior, with K43R alone that was able to keep up with the wild-type and K43T that showed the lowest growth rate. The replication of the experiment in minimal medium (this time only up to a streptomycin concentration of 4'000 μg ml^{-1}) largely confirmed the results obtained in LB, yet the differences in growth behavior between the variants were even more evident (**Figure S2**). K43R was consistently performing better than all other variants at all concentrations tested, although this advantage seemed to decline at higher streptomycin levels. Most noteworthy, K43R was the sole mutant that showed growth rates comparable to the wild-type when no antibiotic pressure was present.

Competition experiments

In order to directly compare the fitness of the various Sm^R variants with that of the wild-type, two direct competition assays were performed by monitoring over time a periodical subcultivation of an initial 1:1 co-culture in minimal medium without antibiotic pressure. The first experiment was shorter (four days) but essentially showed the same trends in populations shift over time. Thus, only the results of the second longer assay are presented here. Samples

were taken one, three, six and eight days after the start of the experiment and the number of total and Sm^R colonies was determined by dilution plate counting (**Figure 3**). The obtained results indicate that three of the variants (i.e., K43N1, K43N2 and K43T) were quickly outcompeted by the wild-type by day one, when the ratio of wild-type to mutant cells already repeatedly exceeded 10:1. After eight days of subcultivation, which we calculated to correspond to about 54 bacterial generations, the final ratio was approximately 1,000,000:1 in favor of the wild-type, conceivably suggesting the unavoidable extinction of these Sm^R variants should the experiment have been prolonged further. A somehow increased fitness compared to the aforementioned variants was displayed by mutant K88R, which exhibited a slower decline resulting in wild-type to mutant ratios of approximately 3:1 and 100:1 after one and eight days, respectively. On the other hand, under the selected growth conditions, mutant K43R demonstrated a fitness that was largely comparable with that of wild-type strain CFBP 1376, with the wild-type to mutant ratio that remained approximately even until the sixth day (44 bacterial generations) and dropped to about 4:1 after eight days (54 bacterial generations).

SNaPshot® assay

The developed SNaPshot® multiplex PCR was validated on single colonies of wild-type *E. amylovora* CFBP 1376 and its Sm^R derivative variants K43R, K43N1, K43N2, K43T and K88R, demonstrating that the method is capable to simultaneously interrogate the three positions on the *rpsL* gene where the base substitutions leading to Sm^R are known occur, thereby discriminating all six variants of *E. amylovora* examined (**Figure 4**).

Relative frequency of spontaneous Sm^R variants

A total of 83 spontaneous Sm^R colonies isolated from the overnight cultures in LB broth without antibiotic pressure were analyzed individually using the *SNaPshot*® assay in order to assess their relative rate of appearance. Next to the common mutations resulting in the K43R and K88R genotypes, which were detected at a reciprocal ratio of approximately 3:1, several smaller colonies were isolated that were apparently not different from the wild-type according to the SNaPshot® Multiplex results and yet were nevertheless able to grow in presence on plates supplemented with 100 μg ml^{-1} streptomycin. Sanger sequencing of the *rpsL* gene in these isolates confirmed that no mutation had occurred in codons 43 and 88, but revealed the presence of two new RpsL variants at codons 91 and 92 that were not described in *E. amylovora* before, i.e. P91L (proline to leucine) and G92D (glycine to aspartic acid) (**Figure**

6). Comparable *rpsL* mutations were previously described in other bacterial species such as *Salmonella enterica* serovar Typhimurium (Bjorkman et al. 1999), *Escherichia coli* or *Thermus thermophilus* (Demirci *et al*, 2013) and were shown to yield a conditional-lethal streptomycin-dependent (Sm^D) phenotype, in which affected bacteria are unable to grow in absence of the antibiotic. The Sm^D phenotype was confirmed for both new variants of *E. amylovora* CFBP 1376 by demonstrating the failure of P91L and G92D to grow in absence of streptomycin in LB and in minimal medium both on plate and in liquid media.

Discussion

Different studies have characterized the molecular basis of streptomycin resistance in *E. amylovora* during fire blight outbreaks worldwide. It is clear that no single mechanism is universally dominant, but rather that the various populations have developed different strategies over the years and at each respective location. High streptomycin resistance, based on chromosomal point mutations in the *rpsL* gene, is known since 1971 (Moller et al. 1972) and was reported as the most frequent mechanism in older studies (McManus et al. 2002), being described for isolates from the western United States and Michigan, New Zealand (Chiou and Jones 1995) and Mexico (de León Door et al. 2013; Smits et al. 2014). Among the variants identified, K43R was the one that was largely prevalent, yet K43N and K43T were found with increased frequency among a set of *E. amylovora* orchard isolates from Oregon (Chiou and Jones 1995). On the other hand, Tn5393-determined resistance was first described in Michigan in 1990, where it is currently considered the prevalent type of resistance (McGhee et al. 2011), and has since been described in several other states such as New York (Russo et al. 2008) and California (Förster et al. 2015). The reason for the prevalence of one general mechanism over the other across the United States is not clear. The chromosomal high-level type of resistance would clearly be favored at streptomycin concentrations that exceed the capacity of the StrA/B aminoglycoside-phosphotranspherases to efficiently inactivate the antibiotic. However, at the recommended on-target concentrations of 50–200 $\mu g\ ml^{-1}$ (Vidaver 2002) both systems are known to be able to effectively protect *E. amylovora*. In this case, other factors such as presence in the environment (i.e., the geographical distribution) and transferability of the Tn5393-element have to be ranked as prerequisites for this type of resistance to be possible at all. Once these condition are met, other effects such as the metabolic burden for maintaining Tn5393 and the growth limitations imposed by the mutation in the S12 ribosomal protein would have to be weighed against each

other to understand their relative fitness penalties at different streptomycin concentrations, which was beyond the scope of this work.

As for the chromosomal mutation, the results obtained in this study indicate that K43R is the only one among the Sm^R variants analyzed with a fitness that is equivalent to that of the wild-type under *in vitro* conditions mimicking the flower environment in absence of selective pressure caused by the presence of antibiotic. On the other hand, K43R did not display an improved resistance to higher concentrations of the antibiotic with respect to the other K43 variants, which were all basically equally capable to grow without particular difficulties at least up to 16'000 μg ml^{-1} streptomycin, yet its growth was generally faster at all streptomycin concentrations tested. Despite the ability to grow at the same high streptomycin levels, variant K88R generally exhibited a slower growth rate with respect to K43 mutations that was inversely correlated to the amount of antibiotic present in the growth medium. This suggests that K88R might be unfavored with respect to all other K43 mutations when the antibiotic pressure is high. Observed trends were similar both in rich and minimal medium, but the differences among variants were more noticeable in minimal medium, when the bacteria have less resources available. This was confirmed in the co-inoculation experiments, in which only K43R was able to maintain a number of CFU that was comparable to the one of the wild-type over several rounds of co-cultivation without Sm pressure, while other variants tended to be quickly outnumbered. This suggests that, once established in the orchard following a streptomycin treatment, variant K43R then possesses the best prerequisites to endure, even if the wild-type is also present, prolonged periods without antibiotic pressure. Conversely, all other variants will be prone to be quickly outcompeted and disappear in absence of streptomycin, so that their presence in the field may be suggestive of an enduring selective pressure.

Assessment of the relative frequency of spontaneous DNA base substitutions leading to streptomycin resistance showed that most mutations resulted in variants K43R and K88R at relative rates of 60.2% and 14.5%, respectively. The underlying base substitutions are in both cases transitions, in which a purine nucleotide is substituted by another purine nucleotide (A → G). This kind of mutations, involving bases of similar shape, are known to generated at higher frequency than transversions (i.e., interchanges of purine for pyrimidine bases or vice versa) (Collins and Jukes 1994). Unexpectedly, two previously unknown variants of *E. amylovora*, P91L and G92D, both leading to streptomycin-dependent phenotypes were also isolated during the experiment, with the incidence of variant P91L that was even higher

(19.3%) than that of K88R. In the case of Sm^D mutants as well, both underlying base substitutions were transitions: a C→T pyrimidine-to-pyrimidine replacement for P91L and a G → A purine-to-purine exchange for G92D (**Figure 6**). These mutation were never detected in the field and are expected to disappear as soon as antibiotic pressure is removed from the system. No mutations involving transversions were detected, suggesting that the corresponding variants (i.e., K43N and K43T) may naturally be generated at lower frequencies.

In summary, our results indicate that all chromosomal *rpsL* mutations investigated lead to comparable levels of very high resistance to streptomycin, which concentration is probably not a major selection factor. Random mutations preferably produce genotypes that are the results of base transitions. Natural selection acts then on the ability of the nascent variants to compete with the wild-type under different growing conditions. The prevalence of variant K43R among streptomycin resistant isolates retrieved in the orchards is attributable to its general fitness, which was superior to that of all other variants under all the conditions tested. It is thus possible that repeated applications of streptomycin can select for different Sm^R variants in the orchard, but that only K43R can then successfully persist for longer timeframes once the selective pressure is removed.

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Table 1. List of *E. amylovora* mutants included in this study.

Strain	Sequence codons 43 / 88	Amino acid substitution
CFBP 1376 wild-type	AAA / AAA	None (Lysine)
CFBP 1376 K43N1	AA**C** / AAA	Lysine to Asparagine
CFBP 1376 K43N2	AA**T** / AAA	Lysine to Asparagine
CFBP 1376 K43R	A**G**A / AAA	Lysine to Arginine
CFBP 1376 K43T	A**C**A / AAA	Lysine to Threonine
CFBP 1376 K88R	AAA / A**G**A	Lysine to Arginine

Table 2. Primers used for *rpsL* amplification and the SNaPshot® assay. Lower case letters represent bases that do not anneal with the template, but that were added to the primers in order discriminate the different SNaPshot® products by size.

Primer	Length	Sequence (5'-3')	Notes/Target SNP
***ErpsL*-fw**	20 bp	GCTCAGCCCTAAAATTCTGC	*rpsL* amplification
E*rpsL*-rev	20 bp	GGCCTTACTTAACGGAGAAC	*rpsL* amplification
***rpsL*_88r**	21 bp	TAACGCACACCTGGCAAGTCT	Codon 88 - 2nd base
***rpsL*_43f**	33 bp	cacatatacCTCGTGTGTACACGACTACCCCTA	Codon 43 - 2nd base
***rpsL*_43r**	34 bp	gtgctgtgtgaaatgGCAGTGCGGAGTTCGGTTT	Codon 43 - 3rd base

Table 3. Optimized composition of the SNaPshot® multiplex PCR. With respect to the manufacturer's recommendation most concentrations were adjusted to various extent. 5x BigDye™ Terminator Sequencing Buffer (Thermo Fisher Scientific), which is not included in the kit, was added in order to compensate for the lower buffer strength resulting from the higher dilution of the SNaPshot® Multiplex Mix.

Component (concentration)	Volume	Final concentration
SNaPshot® Multiplex Mix (2x)	0.625 µl	0.25x
Primer mix:		
***rpsL*_43r_34bp (10 µM)**	0.33 µl	0.66 µM
***rpsL*_43f_33bp (10 µM)**	0.08 µl	0.16 µM
***rpsL*_88r_21bp (10 µM)**	0.08 µl	0.16 µM
Template	1.5 µl	-
BigDye Terminator Sequencing Buffer (5x)	1.25 µl	1.25x
PCR grade water	1.135 µl	-
Total volume	5 µl	

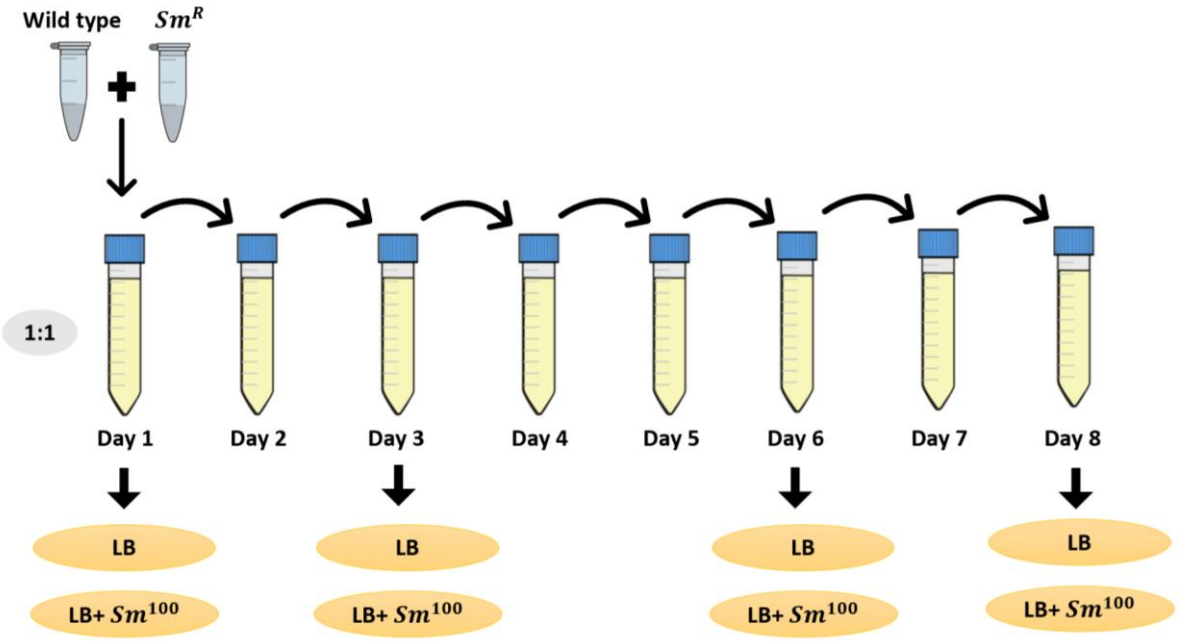


Figure 1. Subcultivation of 1:1 co-cultures of *E. amylovora* CFBP 1376 wild-type and Sm^R derivatives during 8 days (second competition assay) in minimal medium mimicking the flower environment. CFUs were evaluated by plate counts at predefined intervals and the ratios of wild-type vs. resistant isolates were determined for each variant. Colonies growing on LB agar correspond to the total number of CFU, whereas colonies growing on LB Sm^{100} agar represent the number of CFU of the streptomycin resistant variant. The difference between these two number represent the CFU corresponding to wild-type *E. amylovora* CFBP 1376.

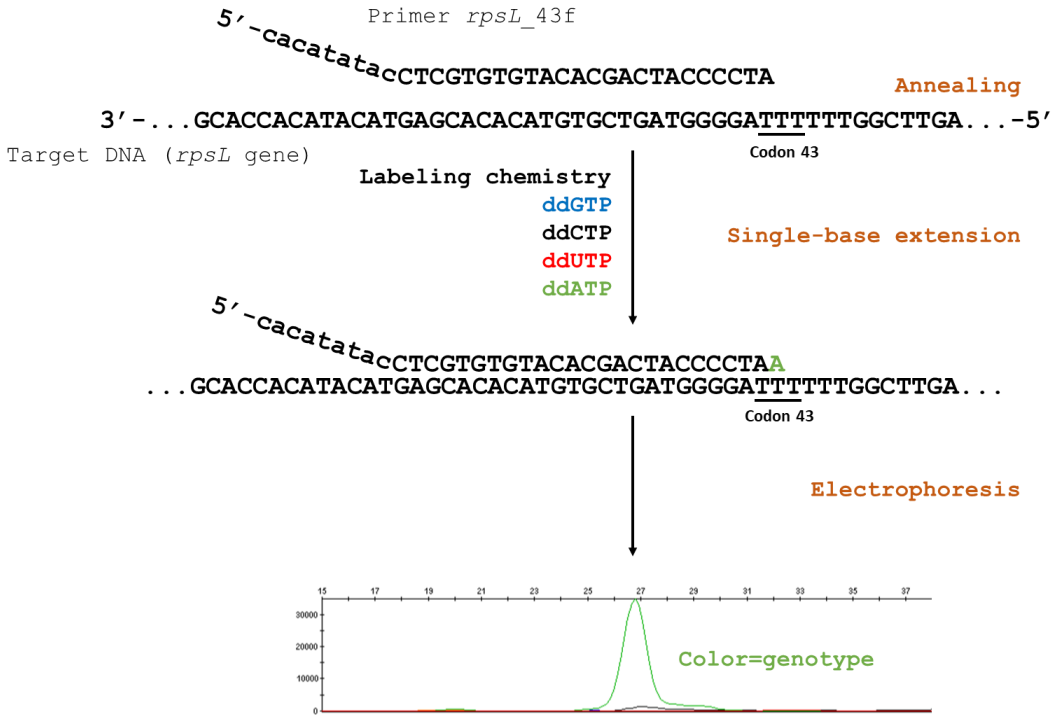


Figure 2. SNaPshot® labeling chemistry relies on single-base extension and termination to detect SNPs immediately downstream of the primer annealing site. Here, primer *rpsL*_43f interrogates the second base of codon 43 of the *rpsL* gene of wild-type *E. amylovora* by binding to the complementary template in the presence of fluorescently labeled ddNTPs and DNA polymerase. The polymerase extends the primer by one nucleotide, adding a single ddATP to its 3′-end. The fluorescence

color readout reports which base was added. The length of the non-annealing 5'-end nucleotide sequence of the primer is tailored to allow size discrimination among the different products of the multiplex reaction.

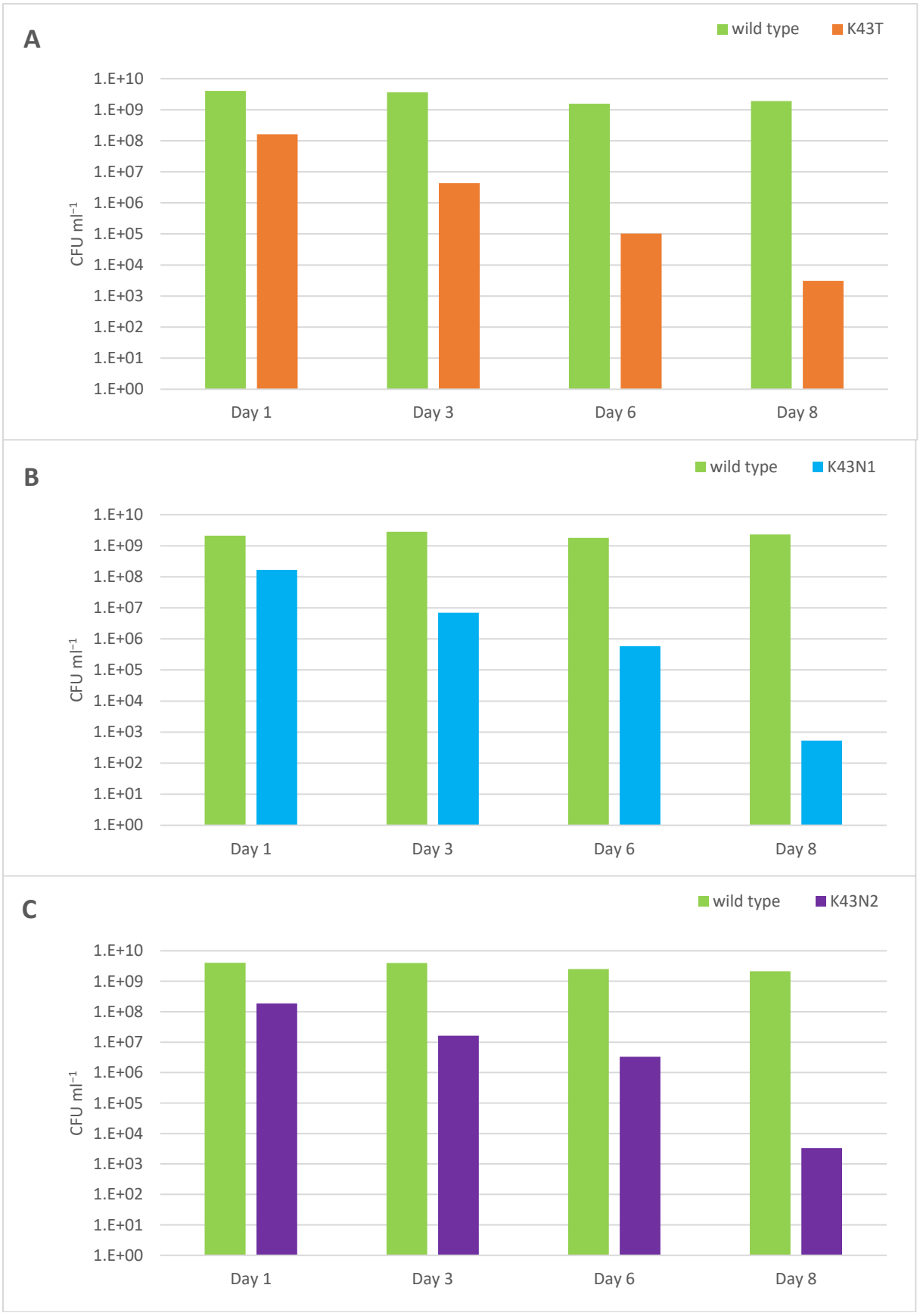
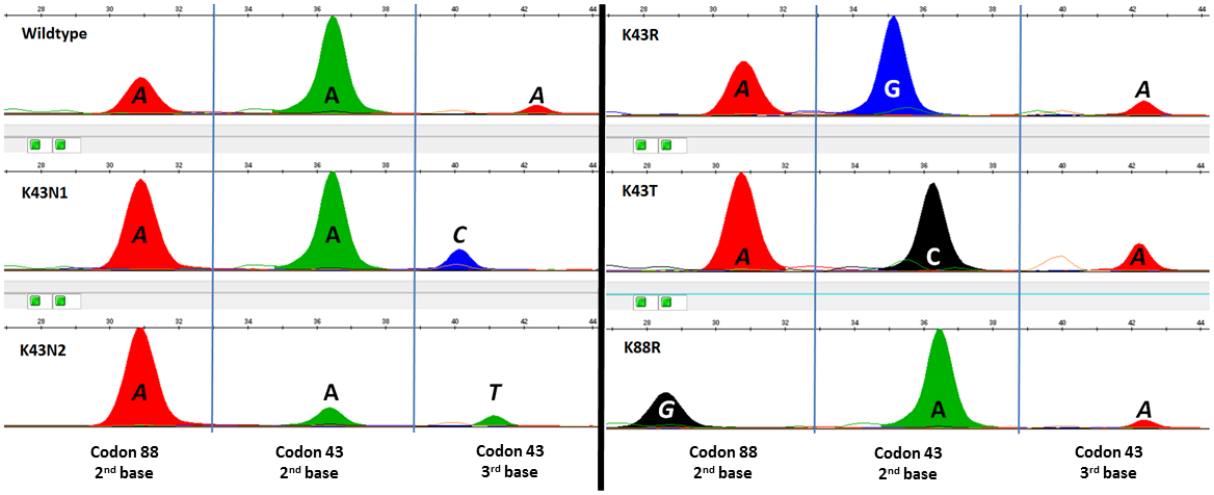

A
wild type
K43T
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
CFU ml⁻¹
Day 1
Day 3
Day 6
Day 8
B
wild type
K43N1
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
CFU ml⁻¹
Day 1
Day 3
Day 6
Day 8
C
wild type
K43N2
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
CFU ml⁻¹
Day 1
Day 3
Day 6
Day 8

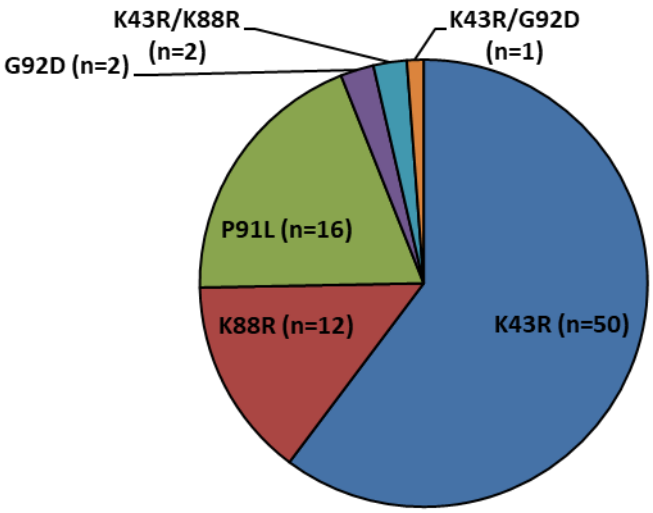
Figure 3. colony counts (CFU ml^{-1}) of wild-type *E. amylovora* CFBP1376 and five different Sm^{R} variants as measured at the end of daily subcultures derived from an initial co-culture inoculated with a 1:1 mixture of the respective strains. **A)** Wild-type vs. K43T; **B)** Wild-type vs. K43N1; **C)** Wild-type vs. K43N2; **D)** Wild-type vs. K88R; **E)** Wild-type vs. K43R. Only variant K43R was able to compete with the wild-type in absence of selective pressure by the antibiotic. K43N1, K43N2 and K43T start to wane quickly and are soon outstripped by the wild-type. K88R declines more slowly, but is nonetheless outnumbered by a factor 100 after eight days.



410

411 **Figure 4.** Genotyping of *E. amylovora* CFBP 1376 variants using the developed SNaPshot® multiplex PCR protocol. The
412 output can be read on the ABI3500 Fragment Analyzer in form of end-labelled products resulting from the extension of each
413 of the primers targeting one of the three SNP locations. The second base of codon 43 is read on the forward strand, while the
414 second base of codon 88 and the third base of codon 43, indicated in *italics*, are read on the reverse strand.

415



416

417 **Figure 5.** Frequency of RpsL streptomycin-resistant genotypes of *E. amylovora* CFBP 1376 arising from an overnight culture
418 in LB broth. Although the K43R variant is widely prevalent in the wild, it represents only 60% of the spontaneous *rpsL*
419 mutations obtained *in vitro*. Two still undescribed variants (P91L and G92D) leading to a streptomycin-dependent phenotype,
420 were also among the spontaneous mutants detected. Three colonies displaying mixed genotypes (K43R/K88R and
421 K43R/G92D) were also detected, suggesting that a second separate mutation occurred on the selective plates containing
422 streptomycin.

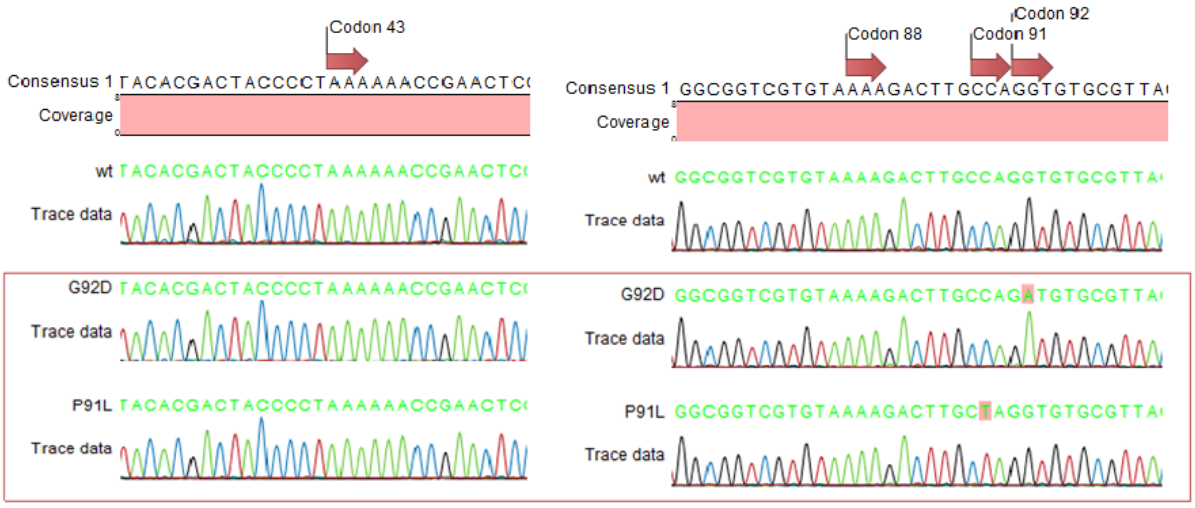


Figure 6. Sequence analysis of *rpsL* gene in streptomycin-dependent colonies and comparison with the wild-type. Streptomycin-dependent colonies display the same sequence as the wild-type in codons 43 and 88, but show point mutations either in codon 91 (CCA→CTA) or in codon 92 (GGT→GAT) leading to proline to leucine (P91L) and glycine to aspartate (G92D) amino acid substitutions, respectively.

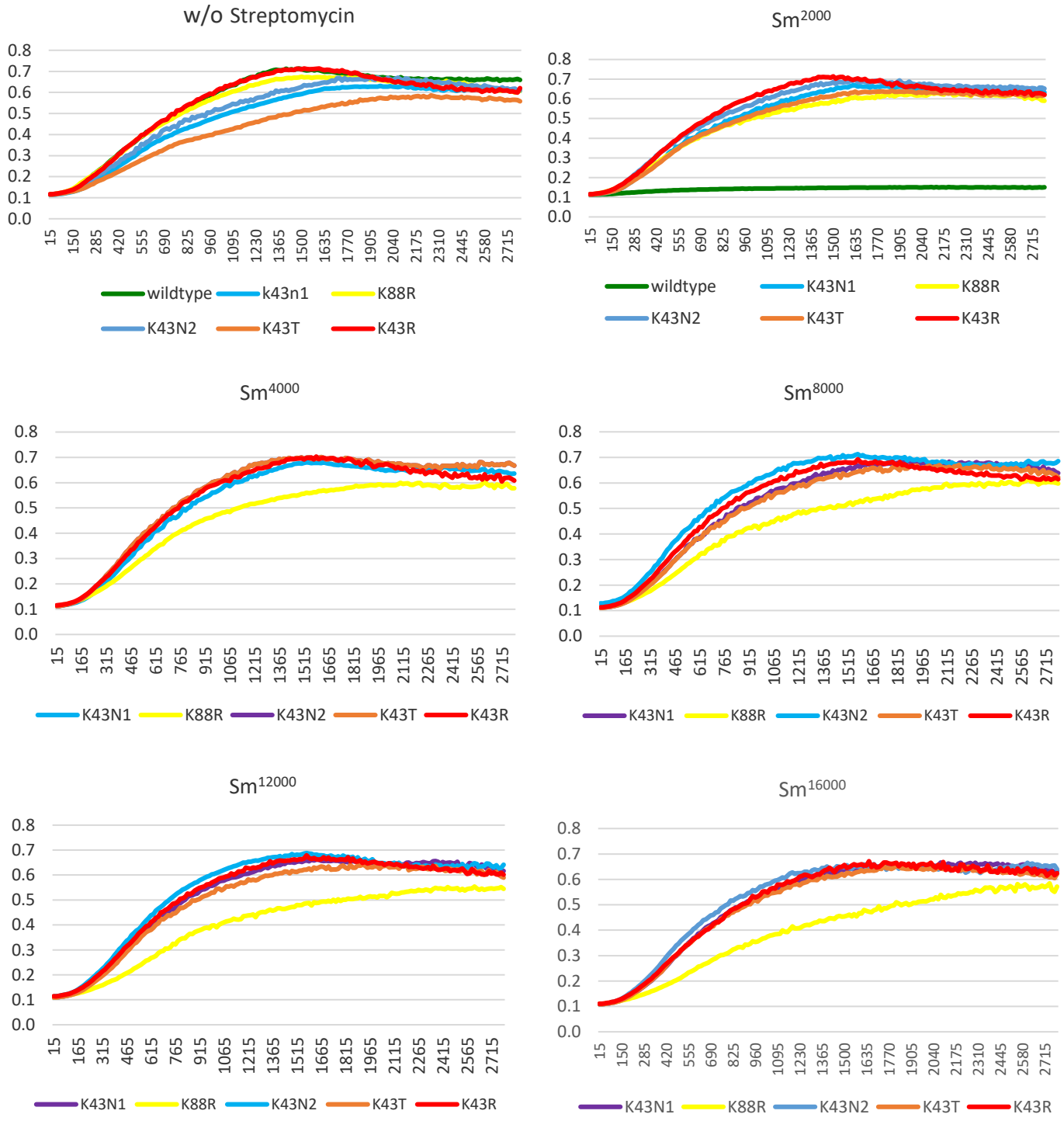
Table S1. Composition flower-mimicking basal medium (modified after Pusey 1999)

K_2HPO_4	**7 g**
KH_2PO_4	**2 g**
Sodium citrate ($Na_3C_6H_5O_7$)	**0.5 g**
$MgSO_4 \cdot 7\ H_2O$	**0.1 g**
Niacin (nicotinic acid)	**0.5 g**
Sucrose	**1 g**
Fructose	**0.5 g**
Glucose	**0.5 g**
Yeast extract	**1 g**
Deionized water	**1 liter**

Table S2. Growth rates of the different variants in rich and minimal medium. in different conditions are summarized in the Average rates calculated by taking the first and final absorbance value of the exponential phase. Growth rate of variant K88R shows a noticeable decrease with increasing streptomycin concentrations that is not noticeable in other variants.

	Rich medium / 28ºC		**Minimal medium / 28ºC**	
	Sm (µg ml^{-1})	**Growth rate (h^{-1})**	**Sm (µg ml^{-1})**	**Growth rate (h^{-1})**
wild-type	**w/o**	0,065	**w/o**	0,055
K43N1	**w/o**	0,052	**w/o**	0,040
	2000	0,061	**100**	0,039
	4000	0,075	**500**	0,044
	8000	0,061	**1000**	0,044
	12000	0,060	**2000**	0,072
	16000	0,060	**4000**	0,074
K88R	**w/o**	0,069	**w/o**	0,059
	2000	0,050	**100**	0,078
	4000	0,046	**500**	0,047
	8000	0,047	**1000**	0,042
	12000	0,043	**2000**	0,023
	16000	0,035	**4000**	0,025
K43N2	**w/o**	0,061	**w/o**	0,039
	2000	0,067	**100**	0,040
	4000	0,067	**500**	0,053
	8000	0,061	**1000**	0,055
	12000	0,064	**2000**	0,055
	16000	0,077	**4000**	0,071
K43T	**w/o**	0,042	**w/o**	0,042
	2000	0,053	**100**	0,043
	4000	0,046	**500**	0,044
	8000	0,046	**1000**	0,059
	12000	0,044	**2000**	0,071
	16000	0,043	**4000**	0,076
K43R	**w/o**	0,062	**w/o**	0,059
	2000	0,064	**100**	0,061
	4000	0,051	**500**	0,053
	8000	0,054	**1000**	0,052
	12000	0,054	**2000**	0,051
	16000	0,063	**4000**	0,071

435 **Figure S1:** Growth curves of *E. amylovora* CFBP1376 and its Sm^R derivatives in LB broth at 28ºC at different streptomycin
436 concentrations.



437

Figure S2: Growth curves of *E. amylovora* CFBP1376 and its Sm^R derivatives in minimal medium at 28ºC at different streptomycin concentrations.