Screening of a High-yield Strain of Avermectin B1a by Colony Analysis In situ

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Abstract

Avermectin, an agricultural antibiotic, is widely used as an agricultural insecticide and an important lead compound of antibiotics. It is manufactured by *Streptomyces avermitilis* through fermentation. Manufacturers pay special attention to screening for strains with high fermentation capacity based on morphological properties of the colony and by the result of shake flask fermentation. These traditional screening methods are time-consuming, labor-intensive and require specialized equipment. Moreover, evaluation of colony appearance is highly subjective. To improve and accelerate the screening process, we developed a rapid *in situ* screening method. Forty-four strains isolated naturally from the spores of industrial high-yielding strains were studied. The data show that the colony fermentation titer is highly correlated with the yield from the shake flask fermentation of avermectin, and the Pearson R is 0.990. The total titer of avermectins by shake flask fermentation is also highly correlated with the B_{1a} titer (Pearson R is 0.994). This result also shows that strains can be quickly screened by analyzing the colony titer. Pigment rings of the colonies that appeared after growing and maturing on the new medium plate were analyzed. The chosen colonies were directly marked and punched and then extracted with methanol. The fermentation ability can be evaluated by measuring the absorbance at 245 nm. This methodology can be applied in both natural breeding and mutation breeding conditions. By continuously breeding from 2008 to 2020, the flask titer of avermectin B_{1a} increased from 4582±483 µg/mL to 9197 ± 1134 µg/mL.

Introduction

Avermectin (Fig. 1) is a class of natural compounds with agricultural antibiotic activity with a unique mechanism of action that can effectively control dipteran, homopteran, coleopteran, and lepidopteran pests and a variety of mites (Danaher et al. 2012). Among them, avermectin B_{1a} shows the highest selectivity and safety. At the recommended dose, it is not only safe for humans and livestock but also innocuous to predators and the environment (Bai and Ogbourne 2016; Wang et al. 2019). Therefore, it is environmentally friendly and has been recommended by the Ministry of Agriculture of China as one of the preferred substitutes for highly toxic pesticides. Recent studies have shown that avermectin also has anticancer, antidiabetic, and antiviral activities and is used in the treatment of several metabolic disorders (Batiha et al. 2020; Pfab et al. 2021). In addition, avermectin can be used as a lead compound for the development of pesticides. The commercialized avermectin products are shown in Figure 1. The 2015 Nobel Prize in Physiology and Medicine was awarded for the discovery and purification of avermectin and artemisinin. Currently, the demand for avermectin B_{1a} in the world is at least 5000 tons annually, which is manufactured by fermentation. A high-yielding strain means lower costs and higher production capacity; consequently, manufacturers are constantly breeding and screening for high-yield strains.

The traditional screening method is to conduct flask fermentation, which is labor-intensive and time-consuming. Moreover, shake flask fermentation requires shaker equipment, which is not suitable for high-throughput screening. Using 96-well plates instead of shake flasks for trial fermentation increases the number of samples that can be screened simultaneously, but it still takes time and effort (Gao et al.

2010a). In comparison to other methods, *in situ* screening is a faster and easier way to identify a high-yielding strain. It screens the strain by the morphological characteristics of the colony and the reaction of the strain with the substrate in the plate (Buffo et al. 2021). A number of different methodologies have been proposed. The typical *in situ* screening technique is the transparent circle method, which was applied to screen for pectinase-producing strains (Abdollahzadeh et al. 2020; Huang et al. 2019), xylanase strains (Yang et al. 2016), and cellulose-degrading strains. By spreading the sensitive bacteria over the agar plate, the inhibition zone is produced *in situ* and becomes a qualitative method to measure antibiotic resistance and industrially to test the ability of solids and textiles to inhibit microbial growth (Bhattacharjee 2015). Spectrum technology has already been used in *in situ* screening. By Raman microscopic and optical tweezers, single cells can be sorted and screened (Nitta et al. 2020; Song et al. 2016; Wang et al. 2013; Yan et al. 2021). By studying fluorescence, single cells can also be picked up easily (Chen et al. 2013; Mutafooulos et al. 2019; Nawaz et al. 2015). The isolation chip (or ichip) is a novel *in situ* method of culturing bacteria (Lodhi et al. 2018; Nichols et al. 2010; Piddock 2015; Sherpa et al. 2015). The soil is diluted in molten agar and nutrients so that only a single cell, on average, grows in the ichip's small compartments or wells (Berdy et al. 2017). These techniques simplify the complicated screening workload, save time, and double the screening volume. This paper is aimed at developing a methodology to screen for high-yielding strains of avermectin *in situ*.

Genetic instability is prevalent in *Streptomyces avermitilis*. It has been reported that 25 of 1300 avermectin-producing strains lost the activity in liquid fermentation (Novak et al. 1993). The manufacturer's strategy is to screen a large number of strains continuously to improve the quality of the strain and prevent it from degenerating. In fermentation plants, the screening protocol is as follows: the diluted spores are cultured on the plating medium for 7-9 days, and then the colonies with ideal appearance and abundant spores are selected for slant cultivation for another 7-9 days. These slants are inoculated and cultured in shaking seed flasks at 28°C for approximately 2 days. The mature seeds are inoculated into fermentation flasks and fermented in a shaker for 8-10 days. After fermentation, broth samples are extracted with organic solvent and analyzed by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC). This screening method is rather subjective, time-consuming, labor-intensive and requires much equipment. Instead of using flask fermentation, a high-throughput screening technique using 96-well plates was developed, which eliminates a significant proportion of shaking and increases the screening scale but is still time-consuming (Gao et al. 2010a). In this paper, Atmospheric Room Temperature Plasma (ARTP) technology was employed to construct a mutation library, which is a novel and rapid mutation tool for microbial evolution engineering (Li et al. 2021; Liu et al. 2020; Qiu et al. 2021; Ye et al. 2021). In combination with the rapid colony analyses, the culture was continuously bred one generation after another in the hope of obtaining a superior production strain.

Materials And Methods

Media and culture

The starting strain of *Streptomyces avermitilis* in this experiment was provided by Hebei Veyong Biochemicals Co. Ltd. (Gaocheng, Shijiazhuang city, Hebei Province, China). Its titer of avermectin B_{1a} in shake flask fermentation was 4582 ± 483 µg/mL. The purity of avermectin B_{1a}, also from Hebei Veyong Biochemicals Co. Ltd., was 99%. ARTP equipment was provided by Tsinghua University (Beijing, China).

The ABK medium used for isolation contained 1.5% glucose, 0.3% beef extract powder, 0.05% dipotassium hydrogen phosphate, 0.05% aspartic acid, and 2% agar. The PBF medium for screening contained 0.56% glucose, 0.2% peptone, 0.1% beef extract paste, 0.1% yeast paste, 0.001% FeSO₄·7H₂O and 2% agar. The strains on ABK or PBF plates were cultured at 28 °C for 10-12 days. Media used for seeding and fermentation in the flask were prepared according to the literature (Gao et al. 2010a; Gao et al. 2010b). The capacity of the shaking flask used for fermentation was 250 mL, and the liquid volume was 25 mL. The flask fermentation was carried out on a constant temperature shaker with large eccentricity and an amplitude of 5 mm.

Mutation and screening methods

The starting strain was cultured on slant medium in a test tube for 7 days. The aerial mycelium was scraped and collected by a sterile shovel and then washed out with 10 mL of sterile water into a sterile flask. The flask (50 mL) contained 15-25 glass beads (approximately 2-3 mm in diameter), creating a simple ball mill. The collected mycelium was shaken and milled in the device for 15 minutes at least 3 times to completely mill and disperse the spores. One hundred microliters of each dilution was spread on a plate for single colony culture. Mycelium was spread on as many plates as available. The strains with abundant spores and obvious melanin circles were selected for trial fermentation.

The strain selected by the above method was cultured on slant medium in a test tube for 7 days again. The aerial mycelium was collected, washed, milled, dispersed and filtrated through qualitative filter paper (pore size is approximately 10 µm). Usually, the OD₆₀₀ of the filtrated suspension was approximately 0.8. High purity helium (99.99%) was used as the working gas of ARTP. The distance between the source of plasma emission and the substrate spore was 2 mm. The working power was 120 W, and the helium flow rate was 12 L/min. The mycelium was processed for 30 s, 45 s, or 60 s. After mutagenesis, the three groups of mutagenized mycelium solutions were suspended in 20 mL sterile water and then fully shaken and diluted 10¹- to 10⁷-fold with sterile water. After gradient dilution, 100 µL of sample from each dilution was plated. Multiple samples from each dilution were used for plating.

Identification of high-yielding strains by colony morphology

The processed spores were cultured on ABK medium or PBF medium at 28 °C for 10 to 12 days. Colonies on the plates were evaluated according to the quantity, size and appearance of spores. Screening for high-yielding strains was performed routinely.

Rapid screening of colonies

The colonies were cultured on plates for 9 days and then punched and transferred to 5 mL methanol after being labeled. After ultrasonic extraction for 10 minutes, the volume was adjusted to 20 mL. The concentration of the solution was defined as the fermentation titer of the colony. Colonies were numbered and sampled for preservation as they grew. Then, they were punched with an aseptic punch and immersed in 5 mL of ethanol. The extraction process was carried out on a shaker at 150 rpm for 30 minutes. The supernatant was obtained by filtration through filter paper with 10 µm pore size, transferred to another tube, and the absorbance at 245 nm was measured to determine the titer of bacteria.

Determination of total avermectins (AVMs)

Ten milliliters of broth was sampled and mixed with 20 mL of methanol, vortexed for 20 minutes to extract avermectin, and then centrifuged at 3000× g for 5 minutes. The supernatant (5 µL) was applied to TCL analysis with 85% methanol in the mobile phase. The spots of avermectin B and A were scraped out and extracted with methanol, and then the extraction was analyzed with a spectrometer by measuring the absorbance at a wavelength of 245 nm.

Determination of avermectin B_{1a} titer

Determination of colony B_{1a} titer: The collected spores were diluted to approximately 10-100 spores/mL, and 100 µL was spread on plate medium to culture for 9 days at 28 °C. Colonies were marked, punched, and the colony blocks were transferred to 10 mL of methanol. After ultrasonic treatment for 15 minutes, the volume was adjusted to 25 mL. The B_{1a} concentration of this sample represents the colony titer and is used to study its correlation with the shake flask fermentation titer.

Determination of flask fermentation B_{1a} titer: Microbes were cultured in the seed flask at 28 °C for 48 hr on a shaker, and 2.5 mL was inoculated into the fermentation flask and cultured for 10 days. To extract avermectin, 1 mL of fermentation broth was sampled and mixed with 19 mL of 95% (v/v) ethanol. Vortexing and ultrasonication were performed 3 times for 5 minutes each. The mixture was centrifuged at 3000 g/min for 15 min. The supernatant was filtered through a microporous membrane (pore size is 0.25 µm, nylon membrane from Unichro) and analyzed by HPLC (Agilent 1100). The detection wavelength was 245 nm, and the analytical column was a C18 column (shim pack, 150 × 6.5 mm). The mobile phase consisted of methanol (42.5%), acetonitrile (42.5%) and water (15%). The flow rate was 1.0 mL/min.

Results And Discussion

Screening *in situ* by pigment ring in PBF plate

The spores of the original strain were diluted and plated for culturing. Colonies were selected for shake flask fermentation. We found that the fermentation ability of offspring was highly variable.

As shown in Figure 2a, b, strains grown on PBF medium plates produced black pigments around the colony, which was absent when cultured on ABK medium plates. Therefore, colonies grown on ABK medium were selected based on the colony size and spore quantity. In addition to these characteristics, the colonies on the PBF medium plates could be selected based on the size and depth of the pigmentation ring. In the avermectin production facility, strain engineers focus on studying and summarizing the relationship between fermentation ability and colony morphology, including growth state, color and spore quantity. Generally, there are three types of colonies of *Streptomyces avermitilis*, namely, the gray powder type, white type and bare type. Among them, the strain with gray powder type colonies usually has the highest production capacity. In contrast, the strains with bare colonies are usually poor in fermentation. On PBF medium plates, 44 strains were selected according to the spore quantity and the black pigmentation ring. Among them, 16 strains fermented 3000-4000 µg/mL avermectin B_{1a}, and only two strains fermented more than 4000 µg/mL B_{1a} (Figure 3), which confirms the genetic instability of *Streptomyces avermitilis* described in the literature (Xi et al. 2020). Without an efficient screening method, the trait of avermectin production will degenerate quickly with passaging.

The correlation between the colony titer on the plate and the shake flask fermentation unit was studied, and the results are shown in Figure 4a. The regression equation is $Y = 3.705X + 122.63$ (Y stands for the shake flask fermentation titer of B_{1a}, X stands for the colony fermentation titer of B_{1a} on the plate), and the Pearson r value is 0.990, indicating a positive correlation. The correlation between the titer of total avermectin and the titer of avermectin B_{1a} was analyzed (Figure 4b), which shows that $AVMs = 11.9 + 2.51B_{1a}$ (Pearson $r = 0.994$). This high correlation means that high-yield strains of avermectin B_{1a} can be identified simply by measuring the total titer of avermectin on the plate.

It has been proven that there is a negative correlation between the production ability of secondary pigments and avermectin. The *avel* gene positively regulates the production of pigments, and deletion of the *avel* gene led to increased biosynthesis of avermectin B_{1a} by approximately 16-fold (Chen et al. 2008). Another study indicated that *avel* functioned as a global regulator in *S. avermitilis* and controlled not only secondary metabolism and morphological differentiation but also primary metabolism. Consequently, on PBF medium plates, colonies with abundant spores, plump morphology and smaller pigmentation rings tend to be high-yielding colonies. However, in the application, the pigment circle is closely related to the growth of the colony (Figure 2). The larger the colony grows, the larger the pigment circle, and the smaller the colony, the smaller the pigment circle. Therefore, it was necessary for us to further develop a relatively objective *in situ* screening method.

Avermectin B_{1a} is usually detected by HPLC, whose pretreatment is tedious, and the process is rather complex, labor-intensive, time-consuming and not suitable for high-throughput screening. Fortunately, the titer of the colony on the plate shows a very close correlation with the titer of the shake flask culture, and

so does the B_{1a} titer and the total titer of AVMs. Therefore, we can develop an *in situ* screening method by analyzing the titer of the colony on the plate.

Rapid screening of colonies

Avermectins are intracellular products that mainly exist within the mycelium of *Streptomyces avermitilis* and very little avermectins are present in the supernatant of the fermentation. However, during fermentation, many brown pigments accumulate in the culture, which have strong UV absorption at 200-300 nm wavelengths. We were concerned that water-soluble substances such as these pigments might be co-extracted by methanol and potentially interfere with the rapid analysis of colonies. Therefore, we analyzed the UV absorption of these water-soluble impurity substances. Fifty milliliters of avermectin fermentation broth was centrifuged at 3000× g for 5 minutes, and the supernatant was analyzed by UV scanning. We found that the maximum absorption wavelength of the impurity was 254 nm (Figure 5). The maximum absorption wavelength of avermectin B_{1a} is approximately 245 nm. The punctured colony with agar was extracted with methanol, and the supernatant of the extraction was also scanned. The data in Figure 6 show that most of the chromatograms had a maximum absorption peak at approximately 245 nm. Two samples lost the ability to produce both pigment and avermectin (Figure 6). The ideal strain should produce only avermectin B_{1a} without producing any pigment at all because these pigments make industrial wastewater hard to treat. In this study, we did not find such an ideal strain in our mutant library. This maximum absorption peak at 245 nm can be used to determine the avermectin content, which can be used to screen for high-yielding strains *in situ*.

The data of *in situ* colony screening are shown in Figure 7, and the colony from the original strain was chosen as a control. The absorbance of the control group was 0.33. Those above the control level were considered the desirable and positive mutants. The majority of mutants were negative and undesirable, and only approximately 6.7% of strains carried the positive mutation. Sample No. 18 showed the highest absorption, reaching more than 0.5, a 51.5% increase compared to the control. This strain was selected and cultured for further study. DNA is a double-stranded structure. Mutations often cause base changes in only one strand, leaving the other strand intact. As a result, there will be at least two genotypes in the same colony. We therefore further diluted the spores of the high-yield strains obtained from the preliminary screening and discarded the heterozygotes to obtain pure high-yield strains. All of the screened strains were studied by flask fermentation, and the results are shown in Figure 8. The flask titer of avermectin B_{1a} of the start strain was only 4582 µg/mL, and that of AVMs was 10587 µg/mL. By screening the library of ARTP-induced mutants, we selected a strain with a flask titer of avermectin B_{1a} of 6137 µg/mL and AVMs of 16919 µg/mL. By secondary screening, the average flask titer of avermectin B_{1a} increased to 7017 µg/mL, and AVMs increased to 17027 µg/mL. The flask titers of avermectin B_{1a} and AVMs were maintained during the next two generations of fermentation, suggesting stable inheritance of this excellent trait in the mutant.

In situ screening technology is widely used in microbiology. It is well known that *Penicillium* was discovered by observing the plate *in situ*. Bacteriostatic circles and transparent circles can be classified as *in situ* screening techniques. In fact, *in situ* screening is often the first choice for screening a special strain because it is fast, intuitive and scalable. However, strains of avermectin are notoriously hard to screen *in situ*, and there was no report of successes prior to this study. We considered two effective methods: one is to examine the pigmentation ring on the PBF medium plate, and the other is to analyze the colony extract. QPix™ Microbial Colony Picker can be applied directly on PBF medium plates. High-throughput analysis of colony extracts can be performed in 96-well plates, combined with background subtraction of 254 nm absorption, to achieve fast and accurate assessment of strain performance.

For industrial manufacturing, an excellent avermectin strain should not only produce more avermectin B_{1a} but also have a high B_{1a}/B_{1b} ratio. In the separation process, avermectin B_{1a} was purified by a recrystallization process. Because of the similarity between B_{1a} and B_{1b} in chemical structure and solubility, if the concentration of B_{1b} is too high, the content of B_{1b} in the product is also high, and B_{1b} is an undesirable impurity in the product. Strains in the paper were separated, and 30 of them were selected for flask fermentation analysis (Figure 9). Although we did not deliberately screen for high B_{1a}/B_{1b} strains, the majority of strains isolated naturally had a higher B_{1a}/B_{1b} ratio than the control group. This may be due to the high B_{1a}/B_{1b} ratio of the starting strain. As a result, despite B_{1b} also having a peak absorption at 245 nm, high B_{1a} strains can still be screened out by this method.

Finally, the inconsistency of colony performance in shake flask fermentation versus in industrial large-scale fermentation should be discussed. In laboratory settings, strains were selected by colony potency analysis and shake flask fermentation tests. However, their high yield and other favorable characteristics are often not reproduced when cultured in large fermenters in industry. We have experienced this phenomenon in our previous work with an avermectin manufacturer (company name). We sent several excellent strains to the plant for trial fermentation. These strains in general are better than the strains they were using, but it is interesting to note that the best performer in the fermenter was often not the strain with the highest performance in the lab; instead, the second highest-producing lab strain was the best in the fermenter (unpublished data). Shaking flasks and fermenters are very different in many aspects. In addition to temperature and nutrient supply, oxygen availability is vital to the growth of microbes and cells in culture. In shaking flasks, the transfer of oxygen takes place via two liquid surfaces, and the efficiency is determined by the size and shape of the flask, agitation speed, filling volume, and ambient conditions. Cultivation processes in shaking flasks are never under absolute control, as they are not monitored in real time for critical parameters such as temperature, dissolved oxygen or optical density. Temperature and dissolved oxygen, for example, can only be regulated by controlling ambient conditions in a sealed shaker system or a cultivation room. Automated processes, such as feeding the culture according to defined profiles or the integration of control loops, cannot be accomplished. The fermenter is equipped with multiple sensors that allow for real-time monitoring. Temperature, dissolved oxygen, pH and biomass are measured constantly and displayed numerically and graphically. Exhaust, metabolites, and redox potential can be calculated based on these measured parameters in the fermenter. Various

types of impellers operating at specific agitation speeds can meet the individual requirements of different cell types regarding shearing force and mixing efficiency. Strains sensitive to shearing force may produce poorly in fermenters but better in flasks. Antifoaming agents are necessary in fermenters to reduce the surface viscosity of the film, increase the velocity of drainage, and enhance the diffusion of gas. However, sensitive strains tend to age easily in the presence of defoamers and strong shearing force, which are likely the causes of loss of genetic stability, persistent poor fermentation and suboptimal performance in fermenters. Therefore, it is necessary to add a certain amount of defoamers, such as soybean oil and silicon polyether, to the media during screening to help select strains with good tolerance to shearing force and defoamers. This could be tested in future studies to improve our screening method.

In support of the significance of shearing force, our lead authors have made the following observation while working for an avermectin manufacturer (unpublished work, Hebei Veyong Biochemical Co., LTD). In 2000, the titer of avermectin was only approximately 1800 µg/mL, and the fermentation process could not continue for a long time because of hyphal aging. When the shaft of a fermenter broke by accident, stirring had to be stopped. The whole fermentation process was carried out only by blowing sterile air. While this fermentation unit was expected to be particularly low due to this accident, we were pleasantly surprised that the fermentation titer of avermectin was drastically improved in subsequent analysis. The hyphae in this particular fermentation broth appeared globular under the microscope because high-speed stirring is not conducive to mycelium ball formation. This suggests that *Streptomyces avermitilis* is sensitive to shearing force, causing the discrepancy between the titer in a flask culture and in a fermenter. This also implies that the airlift fermenter may be better for *Streptomyces avermitilis* fermentation. There have been some previous studies on the effects of stirring on fermentation (Lopez-Ramirez et al. 2018; Zhang et al. 2010). The correlation between the colony titer and the shake flask titer was studied in detail by Xi Cheng (2020, Hebei Xingbai Agricultural Technology Co., Ltd). Eleven single colonies were fermented in shaking flasks, and their potency was analyzed by HPLC. The correlation coefficient was 0.924 between the titer of a single colony and the titer of the shaking flask. At the significance level of 0.05, there was a positive correlation between the single colony titer and the corresponding shake flask titer. Despite the difference in medium compositions, moisture contents, and modes of nutrient transfer, plates and flasks are nonetheless highly correlated in terms of fermentation level.

Conclusions

Streptomyces avermitilis is genetically unstable and easily degenerates. Active strain screening is very important to maintain its fermentation ability. The colony fermentation titer was highly correlated with the shake flask fermentation of avermectin, and the Pearson R was 0.990. The total titer of avermectins from shake flask fermentation was also highly correlated with the B_{1a} titer (Pearson R is 0.994). By analyzing the UV scanning spectrum of the fermentation broth and the spectrum of the methanol extract of the colony, the optimal wavelength for screening was identified. By analyzing the ultraviolet absorption of the colony methanol extract at 245 nm, the strains can be quickly screened *in situ*.

The spores of *Streptomyces avermitilis* were treated with ARTP to establish a mutant library. Avermectin was extracted from the colony with methanol, and the absorbance was measured at 245 nm. A strain with 51.5% higher absorbance was selected. The fermentation ability of avermectin B_{1a} of this strain was as high as 7017 µg/mL, which was an increase of approximately 55.1% compared to that of the starting strain.

Declarations

Contributions

GZX conceived and designed the research and wrote the manuscript. LJH, HF and ZB conducted the experiments. LZX and XTY contributed analytical tools.

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Ethics declaration

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

All authors read and approved the manuscript. The authors declare no competing interests.

DAS

All data generated or analyzed during this study are included in this published article.

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Figures

Fig. 1

Figure 1

Avermectin and its commercial derivatives

Figure 2

Schematic analysis of morphological screening of avermectin-producing strains. (a) Colonies on PBF medium, (b) colonies on ABK medium

Fig. 3

Figure 3

The results of shake flask fermentation analysis of naturally isolated colonies of the starting strain

Fig. 4

Figure 4

Correlation of the fermentation process of the strain. (a) Correlation between plate colony titer and shake flask fermentation titer; (b) Correlation analysis of total titer of avermectin and the B_{1a} of avermectin in shaking flask fermentation results of natural isolation colony

Fig. 5

Figure 5

Analysis of UV scanning chromatogram of the supernatant of fermentation broth

Figure 6

Analysis of UV scanning chromatogram of colony extract

Figure 7

Absorption at 245 nm of methanol extraction of the colonies

Fig. 8

Figure 8

Analysis of shake flask fermentation of *Streptomyces avermitilis* strains

Figure 9

B_{1a}/B_{1b} analysis of natural isolates of selected strains