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ARTICLE

Production of ammonia as a low-cost and long-distance antibiotic strategy by *Streptomyces* species

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氨的产生—抗生素之外链霉菌的另一种抗菌策略

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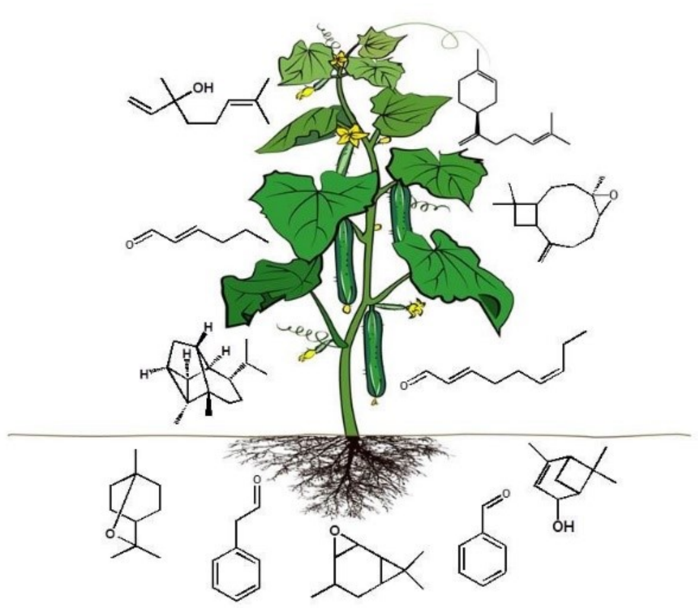
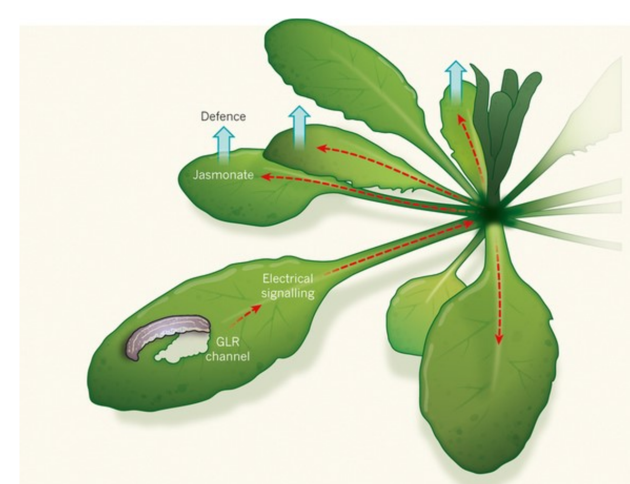
1. Introduction

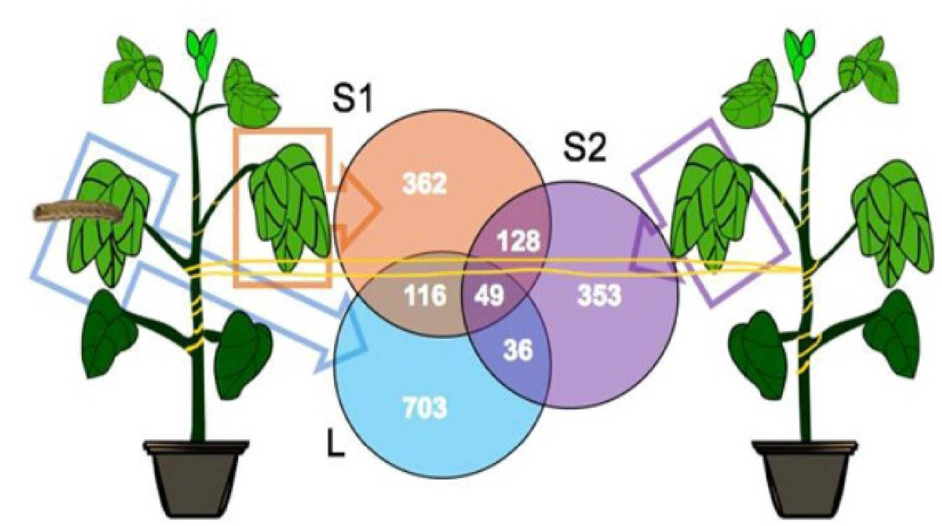
1. Introduction

挥发性化合物（VCs：Volatile compounds）

VCs易通过空气、水或土壤扩散，具有广泛的活性谱，可作为信息化学物质、促生长剂或抑制剂、群体感应和耐药性调节剂，影响其邻居的行为和表型，如应激反应、群体形态、生物膜、毒力和色素沉着等。在土壤中，VCs在种间和种内相互作用中起着重要作用。

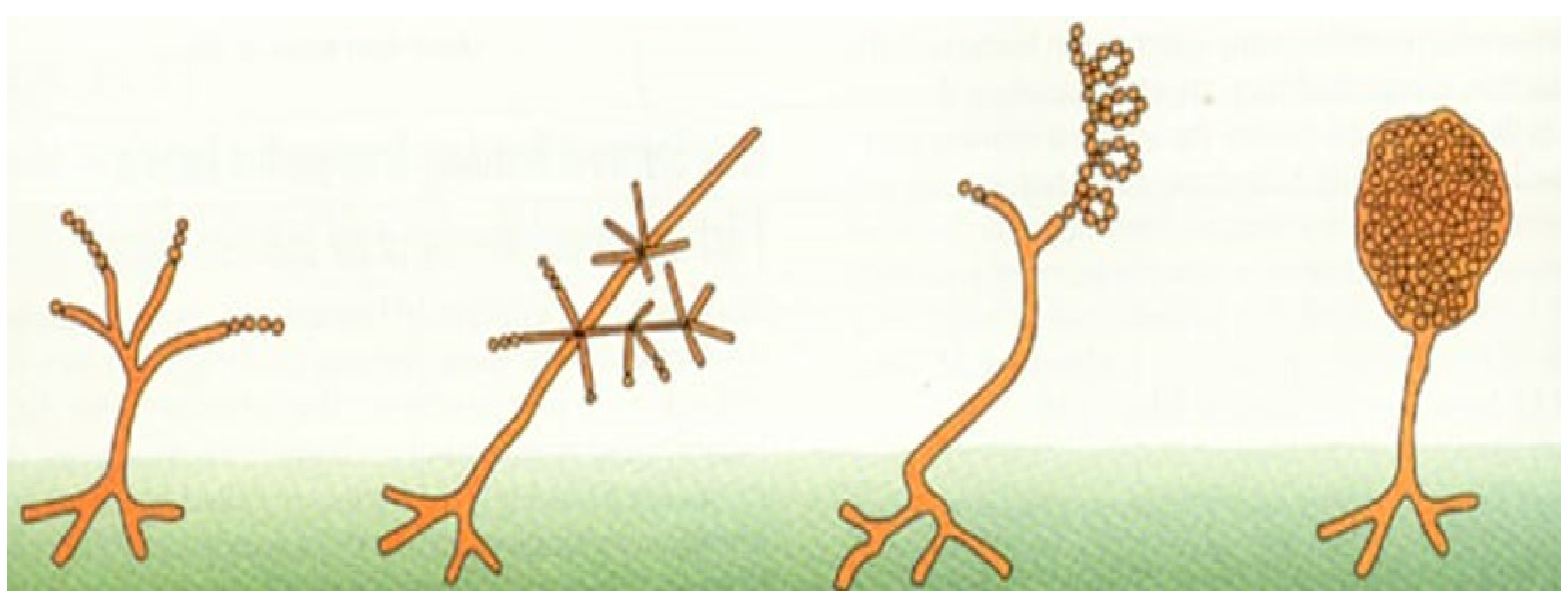


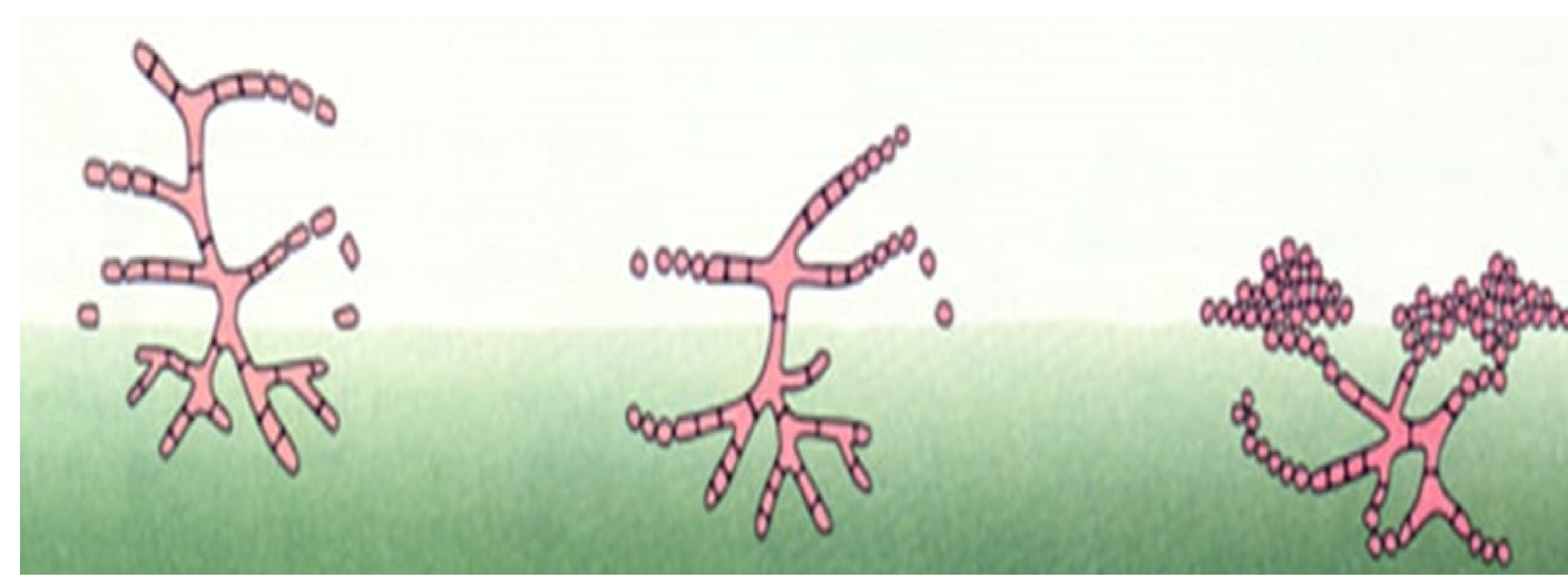




1. Introduction

放线菌为细菌中进化较高级的类群，是土壤中最大的微生物类群之一。被称为自然界的药物制造者，产生具有生物活性的次级代谢产物，可作为抗生素、抗癌制剂、抗真菌剂、驱虫剂和免疫抑制剂。





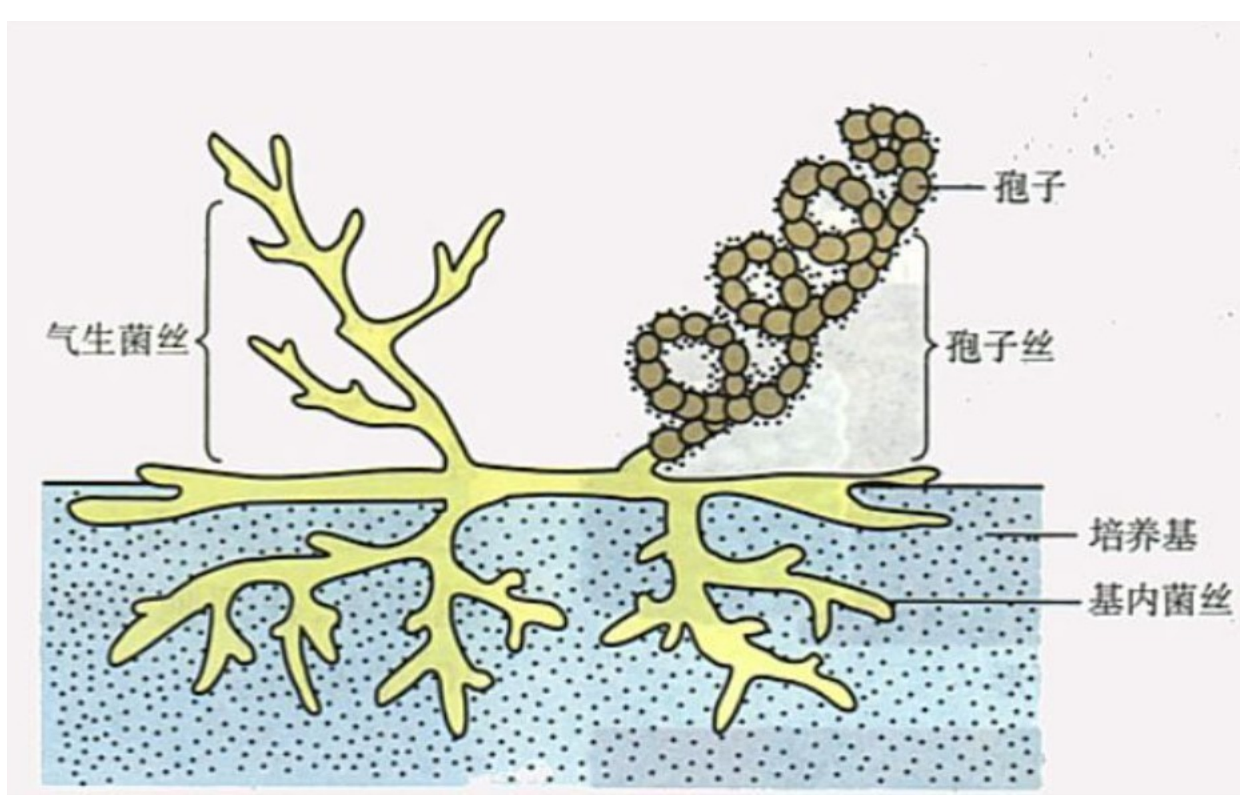
基内（营养）菌丝：摄取营养、产色素

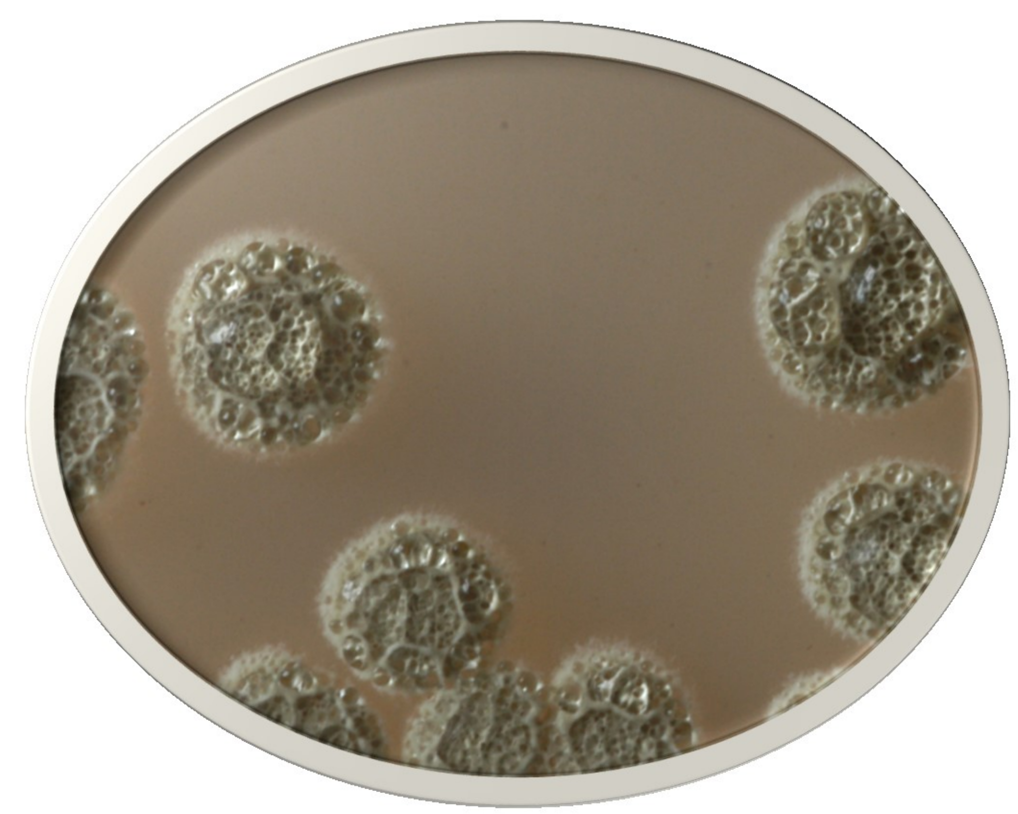
基外（气生）菌丝：吸收氧气、输送营养、产色素

1. Introduction

链霉菌属 (*Streptomyces*) 是放线菌的代表属，也是放线菌目中最大的一个属，是最高等的放线菌。已报道的有千余种，广泛分布于土壤中，对土壤中复杂有机物的矿化发挥重要作用。

放线菌所产抗生素的90%由*Streptomyces*产生，为最重要的抗生素生产菌，如链霉素、四环素、红霉素、新霉素、卡那霉素、井冈霉素等。有的可产生工业用蛋白酶、葡萄糖异构酶或维生素B12等。模式种是白色链霉菌(*Streptomyces albidoflavus*)。





1. Introduction

链霉菌会产生许多挥发性物质（VCs)，目前其生态功能尚未研究清楚。

关于VCs的抗菌活性，主要抗真菌药物活性，其中白色链霉菌(*Streptomyces albidoflavus*)产生的sesquiterpene albaflavenone为细菌中一个特殊典例。

小分子VCs，如H_2S和NO在调节抗生素活性和耐药性中起主要作用：

例如，H_2S的产生可保护细菌免受针对DNA，RNA，蛋白质和细胞壁生物合成的抗生素的侵害。

此外，*Bacillus anthracis*产生NO来保护自己免受氧化应激，并帮助细菌在巨噬细胞中存活，从而在逃避宿主防御中发挥了关键作用。也有研究表明，NO的产生直接保护细菌免受各种抗生素的侵害。

氨通过增加多胺的含量而引起对四环素的抗性，从而导致膜通透性的改变。

还有一些实验证据表明，VCs可能会影响膜的完整性，可能会使细胞对其他破坏细胞的化合物（例如抗生素）更敏感。

细胞间信号的传导？自我保护的武器？

目前，关于细菌产生的VCs的生态作用和VCs抵抗机制的还有待进行研究。

2. Materials and methods

①. Strains, media, culture conditions, and antimicrobial assays

Table S1. Bacterial strains used in the present study.

test microorganisms: grown on LB agar plates

Strains	Description	Reference
E. coli AS19-*rlmA::aph*	Hypersusceptible, KAN^R resistant	Liu and Douthwaite, 2002
E. coli BREL606	Parent strain of *E. coli* AS19	*E. coli* Genetic stock center
B. subtilis 168 枯草芽孢杆菌	Wild-type strain	Barbe *et al.*, 2016
S. coelicolor A3(2) M145	SCP1$^-$SCP2$^-$ wild-type strain	Bentley, 2002
S. lividans 1326	Wild-type strain	Hopwood *et al.*, 1985
S. griseus DSM40236	Wild-type strain	Krainsky, 1914
S. venezuelae ATCC 15439	Wild-type strain	Song *et al.*, 2016
Streptomyces sp. MBT11 Streptomyces sp. MBT21	*Streptomyces* isolates	Zhu *et al.*, 2014
ΔUTR-T	*S. griseus* IFO13350 with deletion of 5'-untranslated region of *gcvTH*	Tezuka and Ohnishi, 2014
GAL61	M145 Δ*gcvP*(::aac(3)IV)	Zhang, Le, 2015 (PhD Thesis)
MBT strains	Actinomycetes collection	Zhu, H. *et al.*, 2014

The ***Streptomyces*** strains were grown on Soy Flour Mannitol (SFM) agar plates to prepare spore stocks. Volatile antimicrobial assays were performed using a **petri dish with two compartments.**

Streptomyces strains were streaked on the SFM side and allowed to grow for **5 days** after which, ***E. coli* or *B. subtilis*** were inoculated on the LB side using a concentration of **10^4 and 10^3 cfu/mL** respectively.

2. Materials and methods

②、Collection and analysis of VCs

VCs produced by *Streptomyces* monocultures grown on SFM agar were collected using a **glass Petri dish** designed for trapping of the volatile headspace. Samples were taken in triplicates from **day 3 to day 5** of growth; after that, the Tenax steel traps were sealed and stored at 4°C until **GC-Q-TOF analysis**.

③、pH change (growth media), ammonia determination, and toxicity

pH值：phenol red indicator 酚红指示剂（0.002%）； after 0, 3, and 5 days.

Ammonia： After 5 days, the ammonia was determined using the Quantofix® ammonium test kit.

Toxicity of ammonia： *E. coli* and *B. subtilis* were incubated in the automated Bioscreen C (Lab systems Helsinki, Finland) in the presence of increasing concentrations of ammonia.

④、Ammonia production in soil

To detect the production of ammonia by *Streptomyces* inoculated in field soil, a microrespirometry-like system（土壤微生物呼吸的测定方法）was used. The system was incubated for 10 days at 30°C.

2. Materials and methods

⑤、HCN（氰化氢）determination

⑥、Whole genome sequencing

E. coli AS19-*RlmA*− and its mutant ARM9; **accession number**: CP027430

⑦、Genetic complementation （遗传互补）of *ompR*（反应调节因子）and *envZ*（感觉激酶） **野生型基因**补偿**突变型基因**的缺陷使细胞表型恢复正常的现象。

⑧、RNA sequencing

For RNA extraction *E. coli* cells were grown to an OD 600 of 0.5.

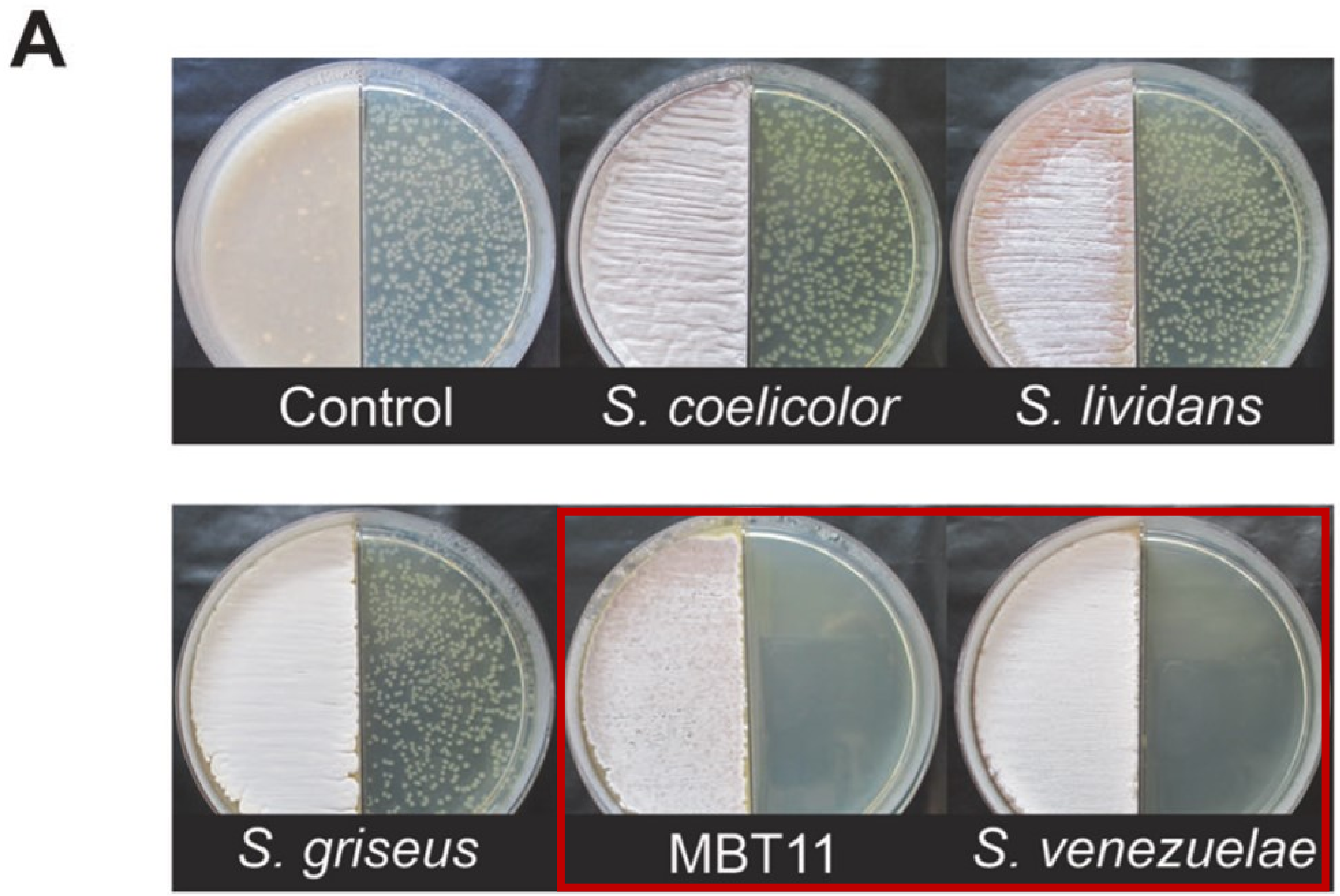
⑨、Synergism of *Streptomyces* AB-VCs with soluble antibiotics

Streptomyces strains were streaked on the SFM side and allowed to grow for **5 days at 30°C** after which, ***E. coli* or *B. subtilis*** were inoculated on the LB side using a culture grown to an **OD=0.5**.

3. Results

①. VCs as bioactive agents in long-distance antibiosis

链霉菌释放的VCs对大肠杆菌ASD19菌株的生物活性研究

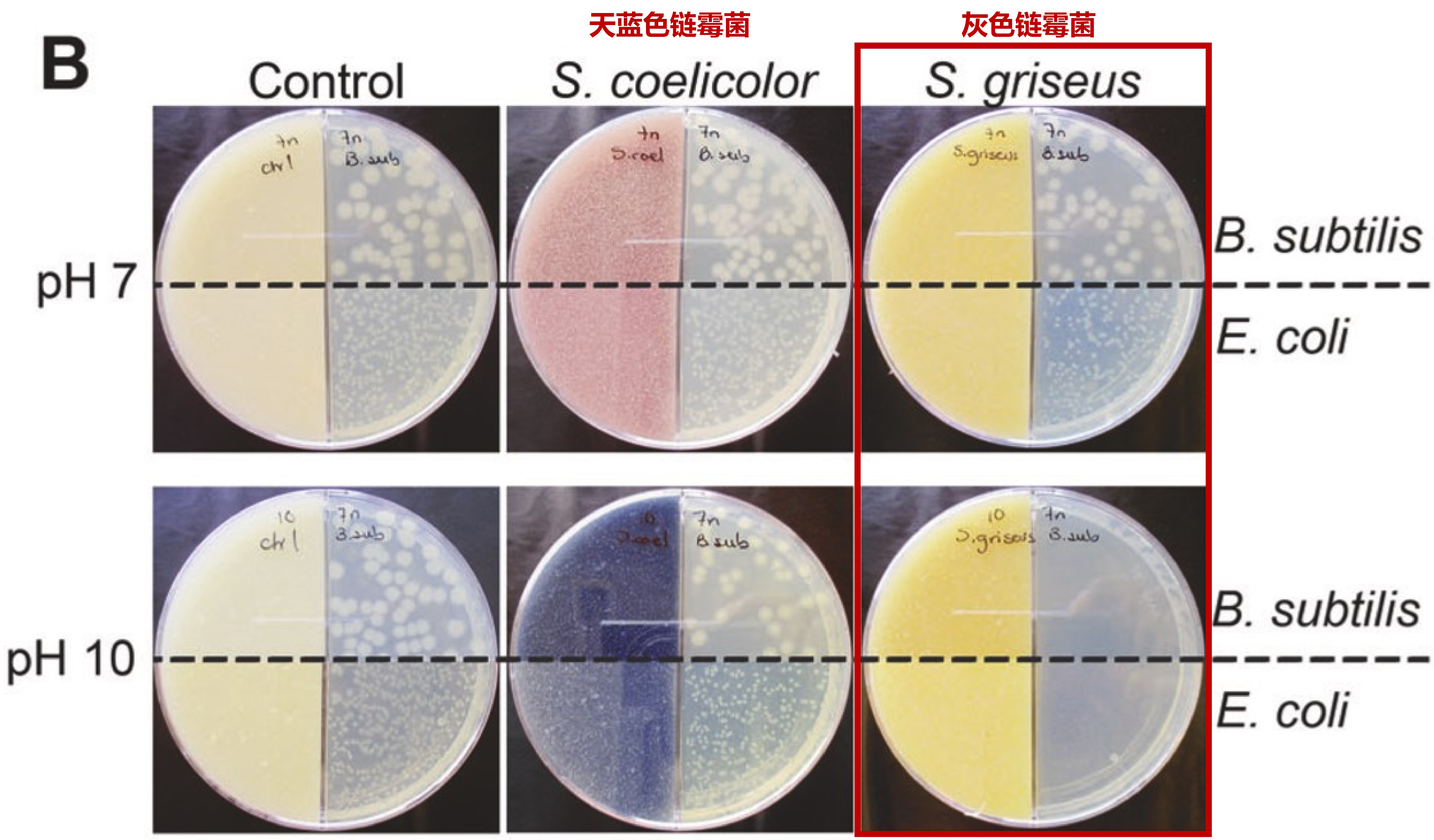


委内瑞拉链霉菌

是否可以通过改变生长条件来引发抗微生物挥发性化合物（AMVC）的产生?

研究表明，pH 10，或添加N-乙酰氨基葡糖，淀粉、蛋白胨、酵母提取物等可提高链霉菌种的抗生素产量。

①. VCs as bioactive agents in long-distance antibiosis



Volatile antibiotic activity of different *Streptomyces* strains grown at pH 7 or pH10, the latter by addition of a **glycine甘氨酸/NaOH buffer**; *E. coli* strain ASD19 was the indicator strain.

①. VCs as bioactive agents in long-distance antibiosis

Table S2. Volatile antimicrobial activity of streptomycetes under different growth conditions. Growth (+) or inhibition (-) of indicator strains *E. coli* or *B. subtilis* is indicated, whereby (+/-) means poor growth, i.e. producing very small colonies.

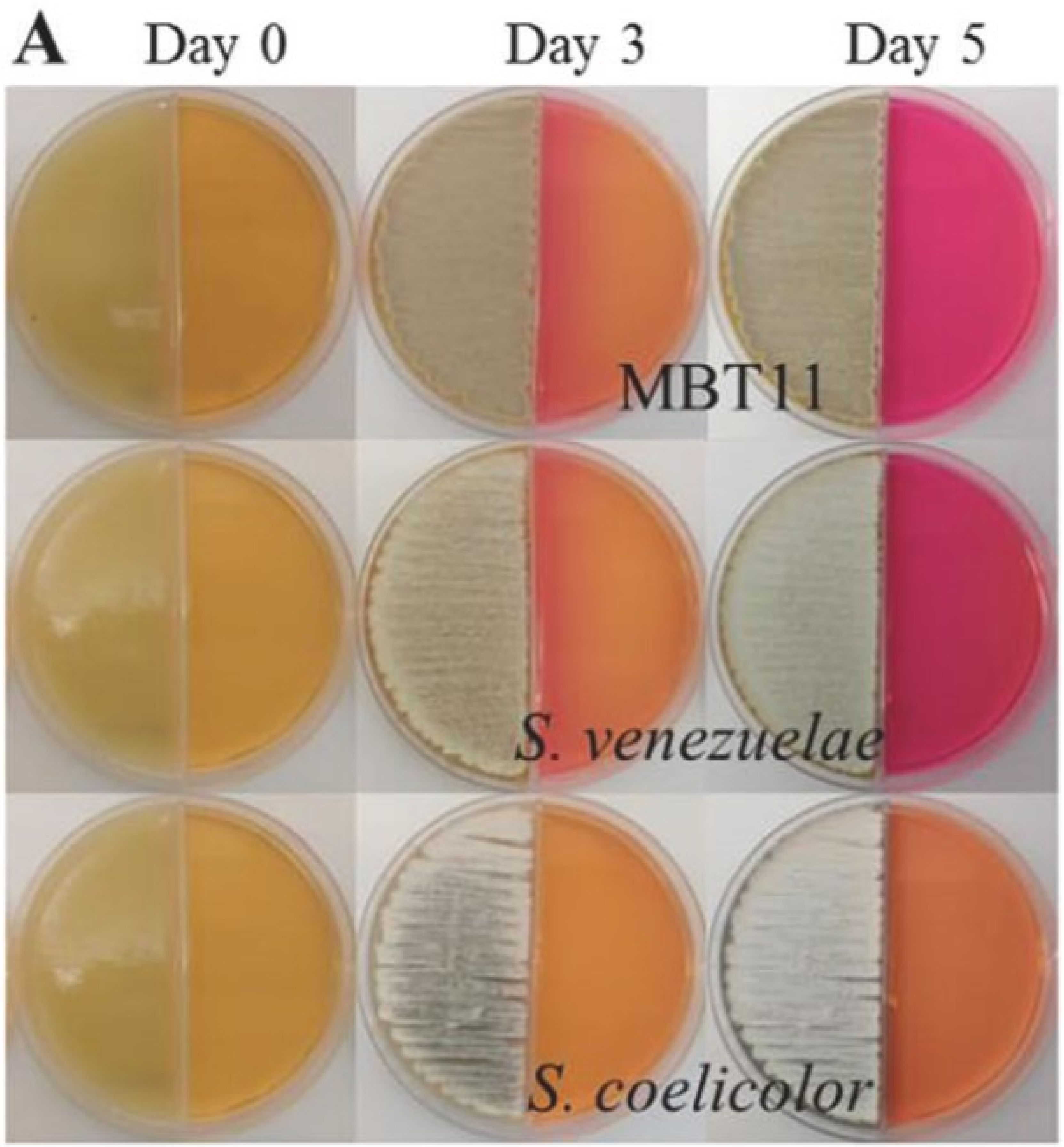
	0.8% peptone		**1% starch**		**MM**	
	E. coli	*B. subtilis*	*E. coli*	*B. subtilis*	*E. coli*	*B. subtilis*
S. coelicolor	+	+	+	+	+	+
S. lividans	+	-	+	+	+	+
MBT11	-	+/-	-	+	+	+
S. venezuelae	-	+/-	+	+	+	+
S. griseus	-/+	-	+	+	+	+

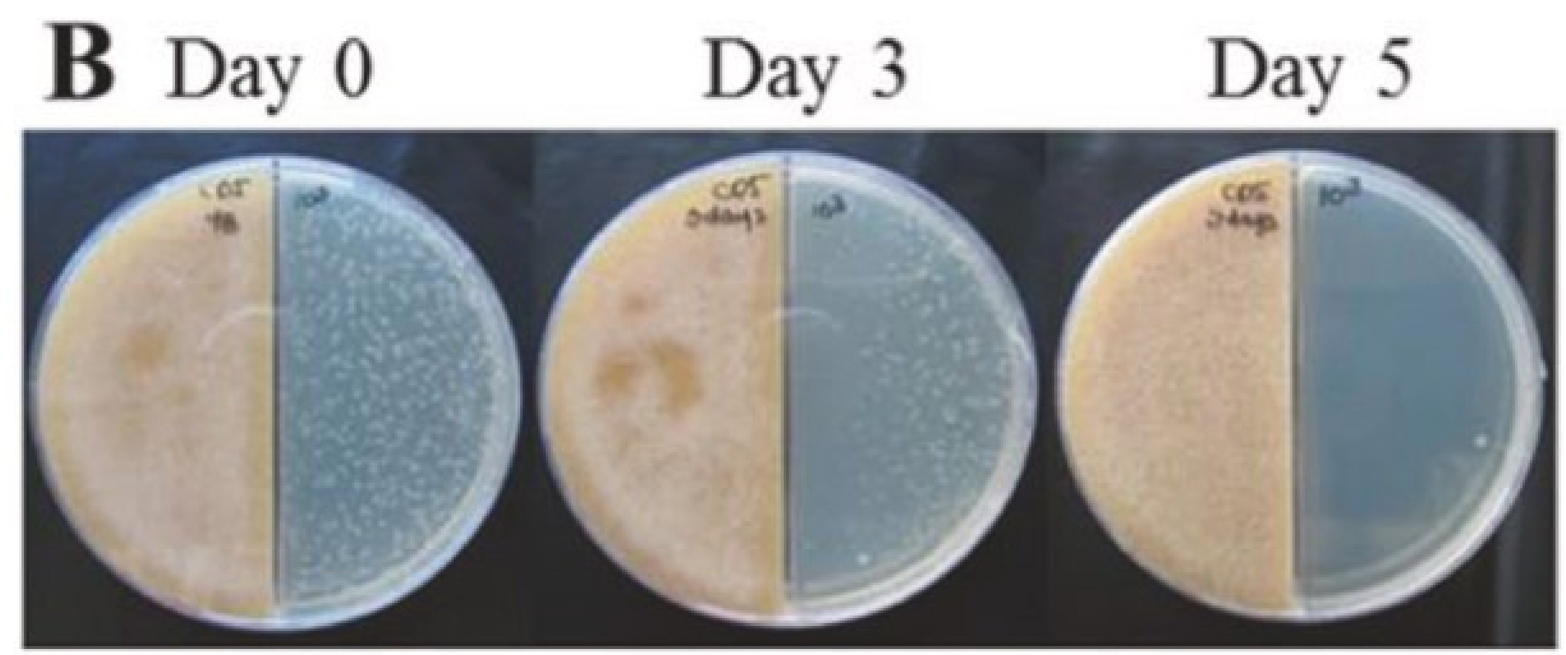
Minimal media (MM) used: L-asparagine 0.5g/L, K_2HPO_4 0.5g/L, $MgSO_4(7H_2O)$ 0.2g/L, $FeSO_4(7H_2O)$ 0.01g/L, Glucose 10g/L, Agar 10g/L.
0.8% peptone or 1% starch were added to MM instead of glucose.

变铅青链霉菌

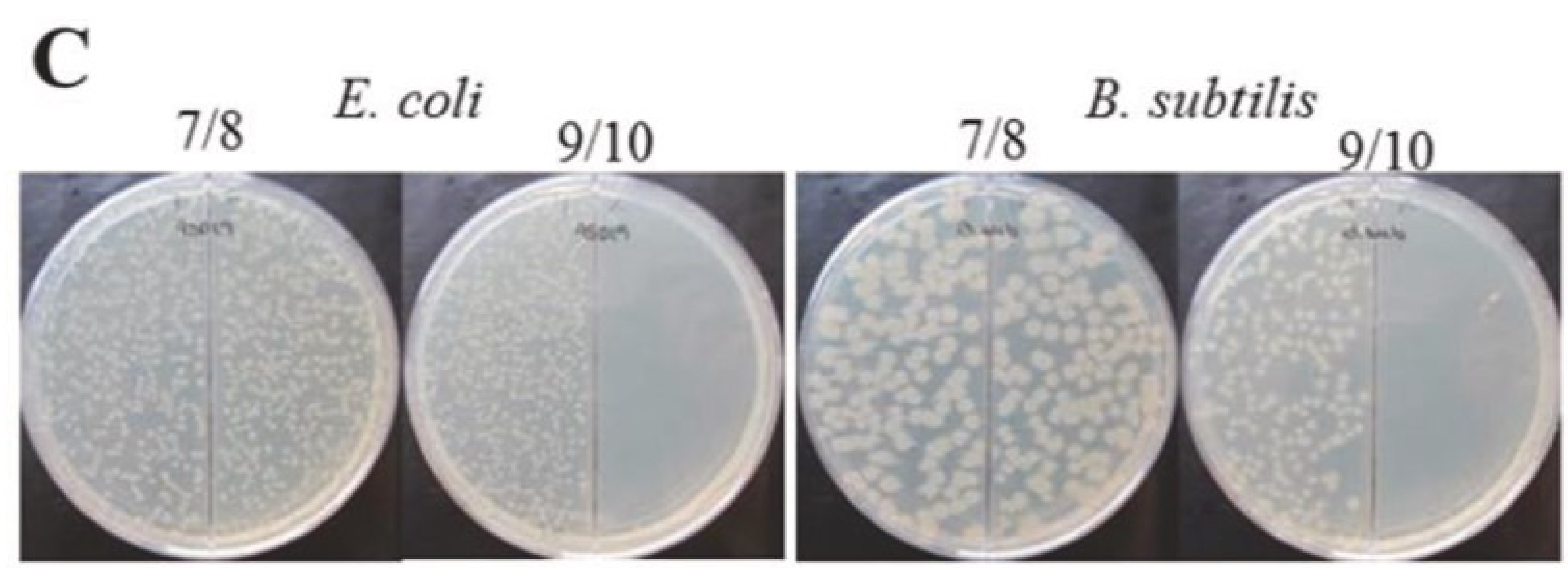
*S. coelicolor*在任何测试条件下均不能产生AMVC。

②. The main inhibitory molecule is ammonia





由于pH的升高，3天后接近链霉菌的大肠杆菌生长受到抑制，而5天后生长被完全抑制。



VCs may induce a change in pH away from the producing colonies. (Jones SE *et al*, Letoffe S *et al.*)

phenol red（酚红）：which changes from pale orange to bright pink when the media becomes more alkaline.

②. The main inhibitory molecule is ammonia

氨、三甲胺（TMA）是可引起pH变化的VCs，根际链霉菌还可产生氰化氢（HCN）。

引起pH变化的主要VCs？

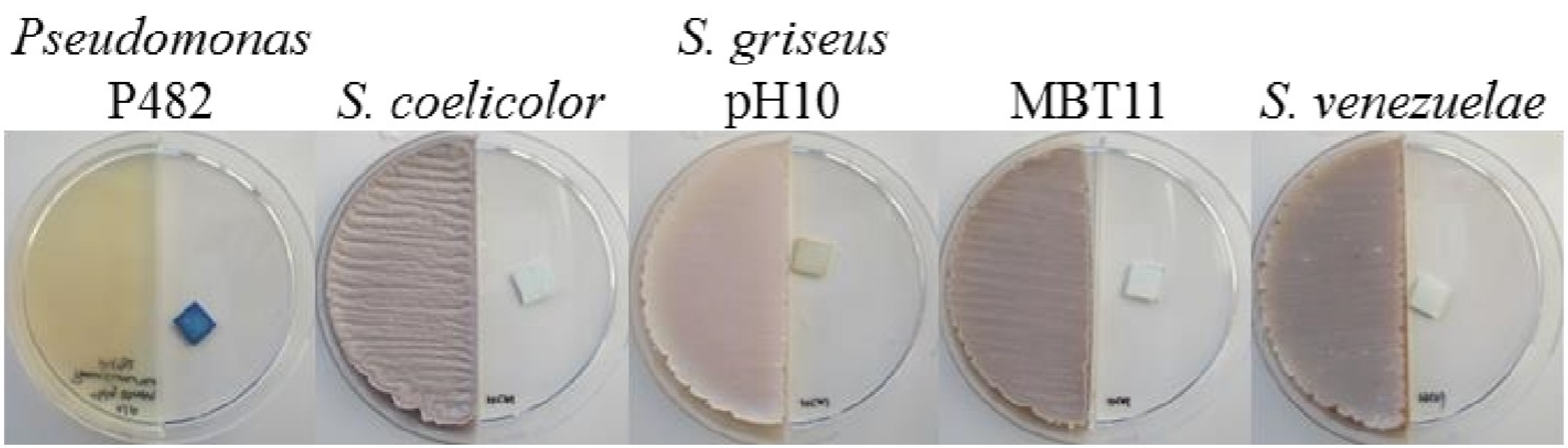


Figure S1. HCN determination from different *Streptomyces* strains. *Pseudomonas donghuensis* P482 was used as positive control[28]. Blue coloration is developed from the oxidation product from HCN + copper(II) ethyl acetoacetate and 4,4'-methylenebis-(N,N-dimethylaniline). none of the *Streptomyces* strains gave a positive reaction showing that the toxicity of the VCs from *Streptomyces* against *E. coli* is not due to the production of the toxic volatile HCN.

②. The main inhibitory molecule is ammonia

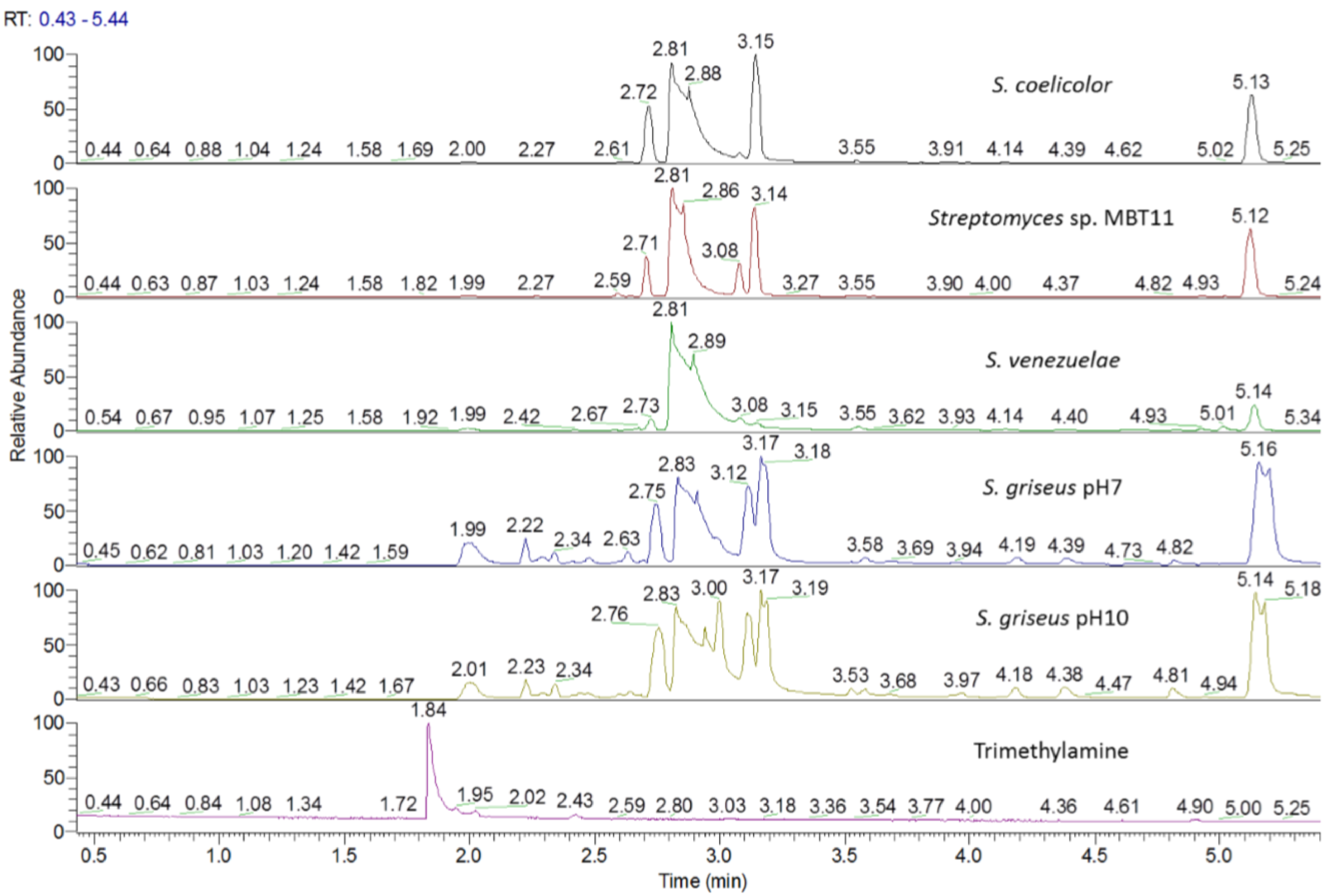


Figure S2. GC-chromatogram showing the absence of TMA in the headspace of *S. coelicolor* (black), *Streptomyces* sp. MBT11 (red), *S. venezuelae* (green), *S. griseus* grown at pH 7 (blue), *S. griseus* grown at pH 10 (yellow-green). Chromatogram of a TMA standard (RT 1.8 min.) is shown below (purple).

②. The main inhibitory molecule is ammonia

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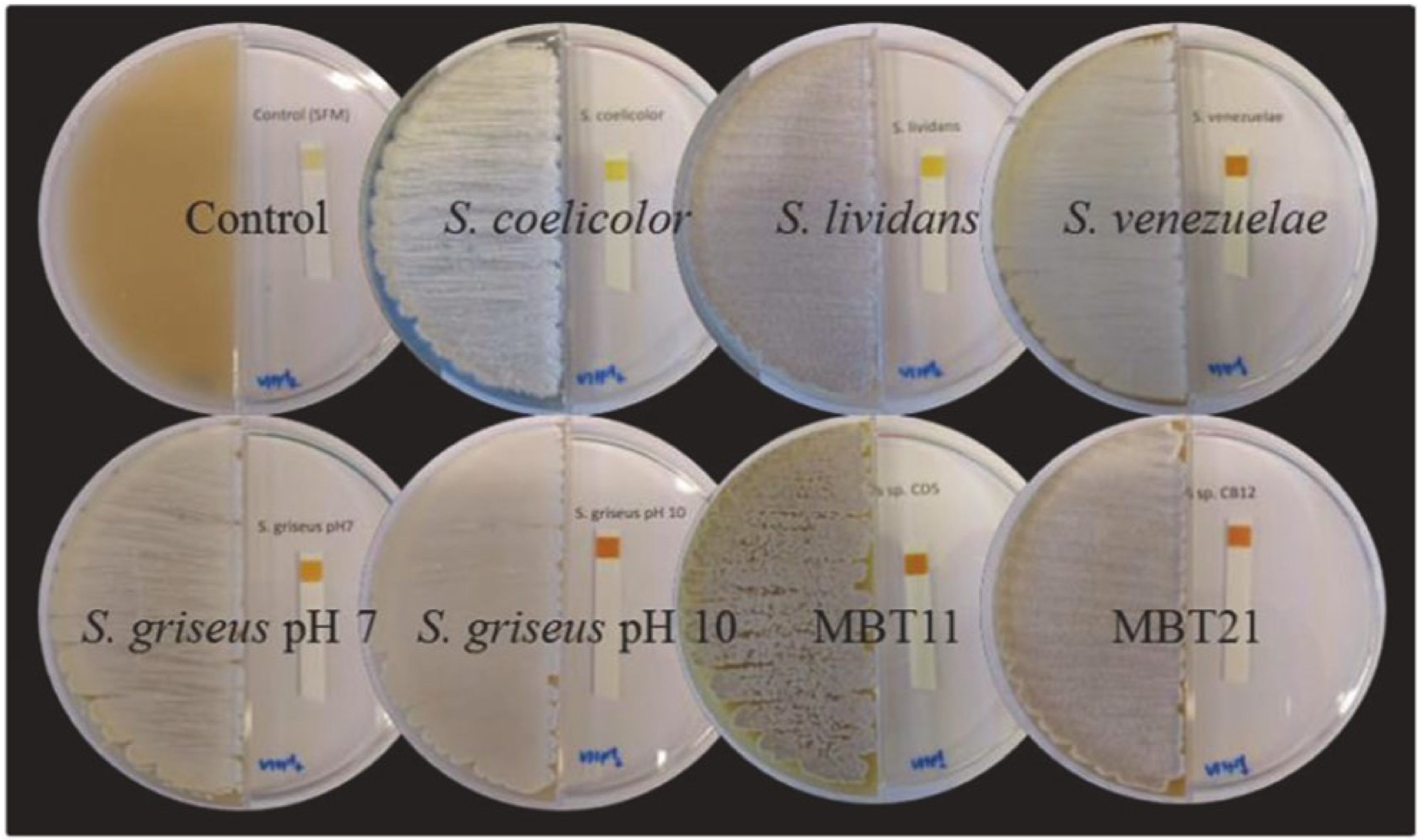
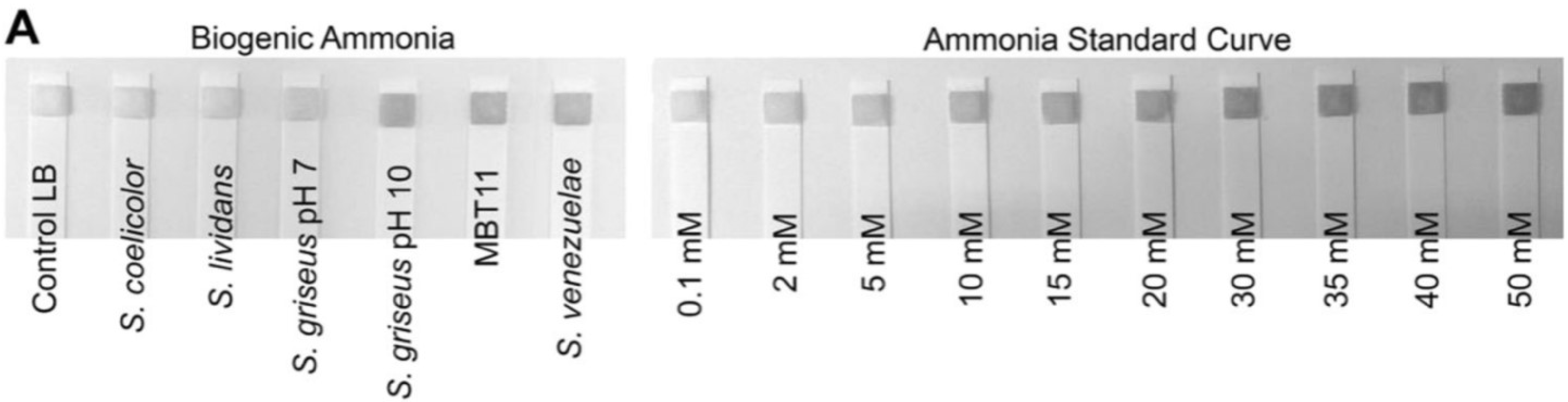


Quantofix® 氨气检测试剂盒

pH升高主要是由于氨引起的；

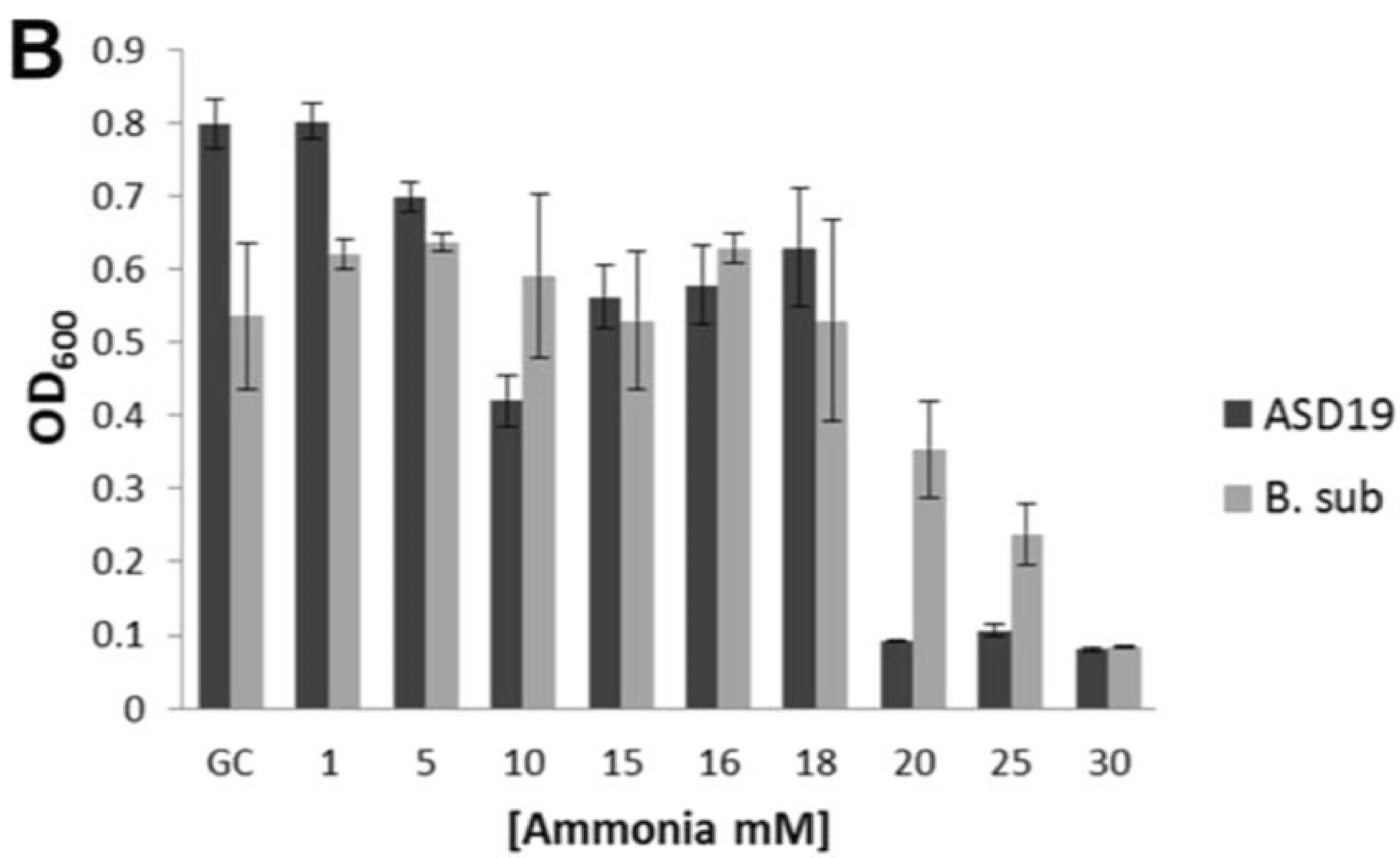
链霉菌的抗菌作用可能与氨气释放密切相关。

②. The main inhibitory molecule is ammonia



暴露于链霉菌VCs的LB琼脂提取物的氨含量。

LB琼脂提取物的氨标准曲线



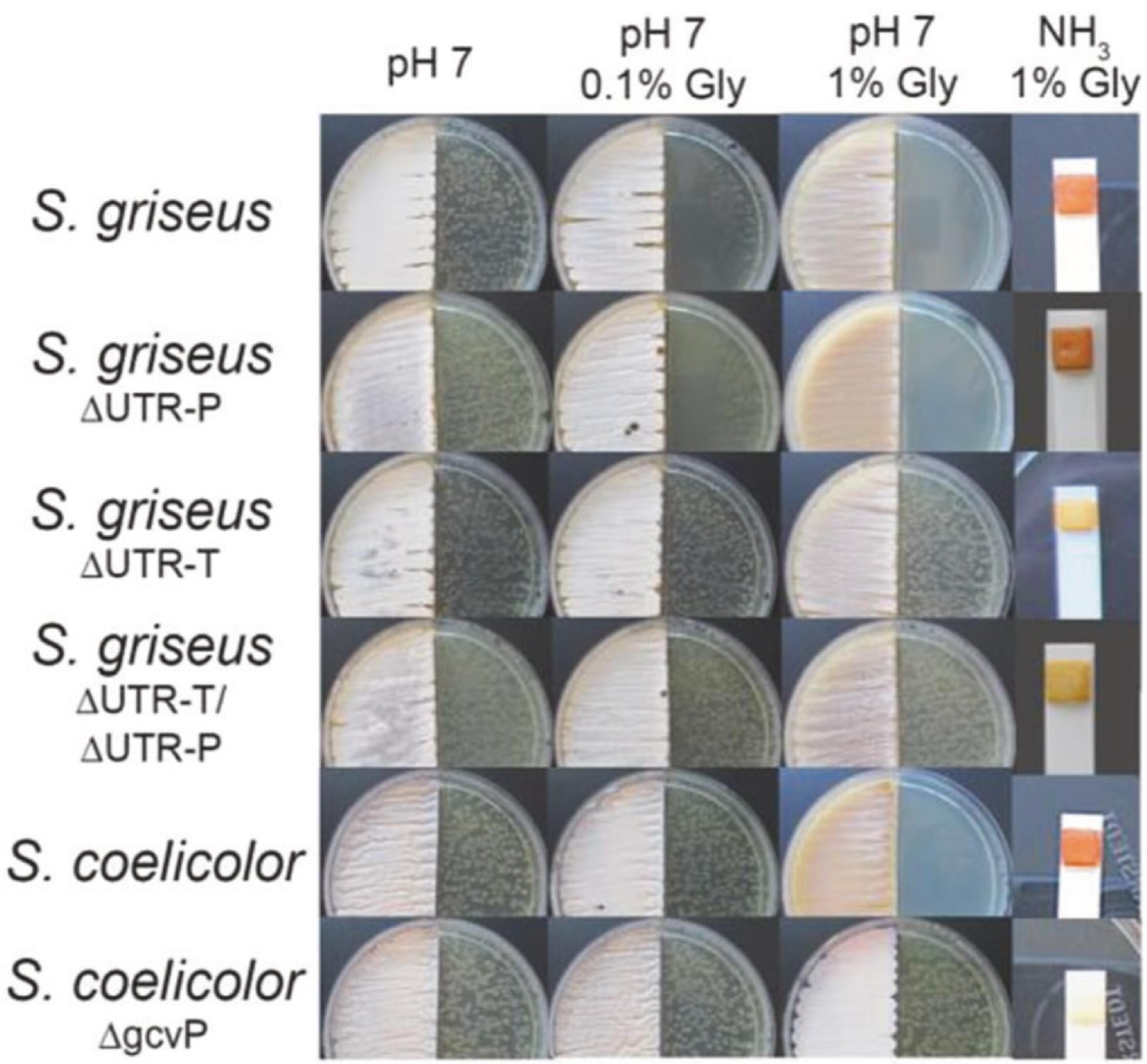
大肠杆菌

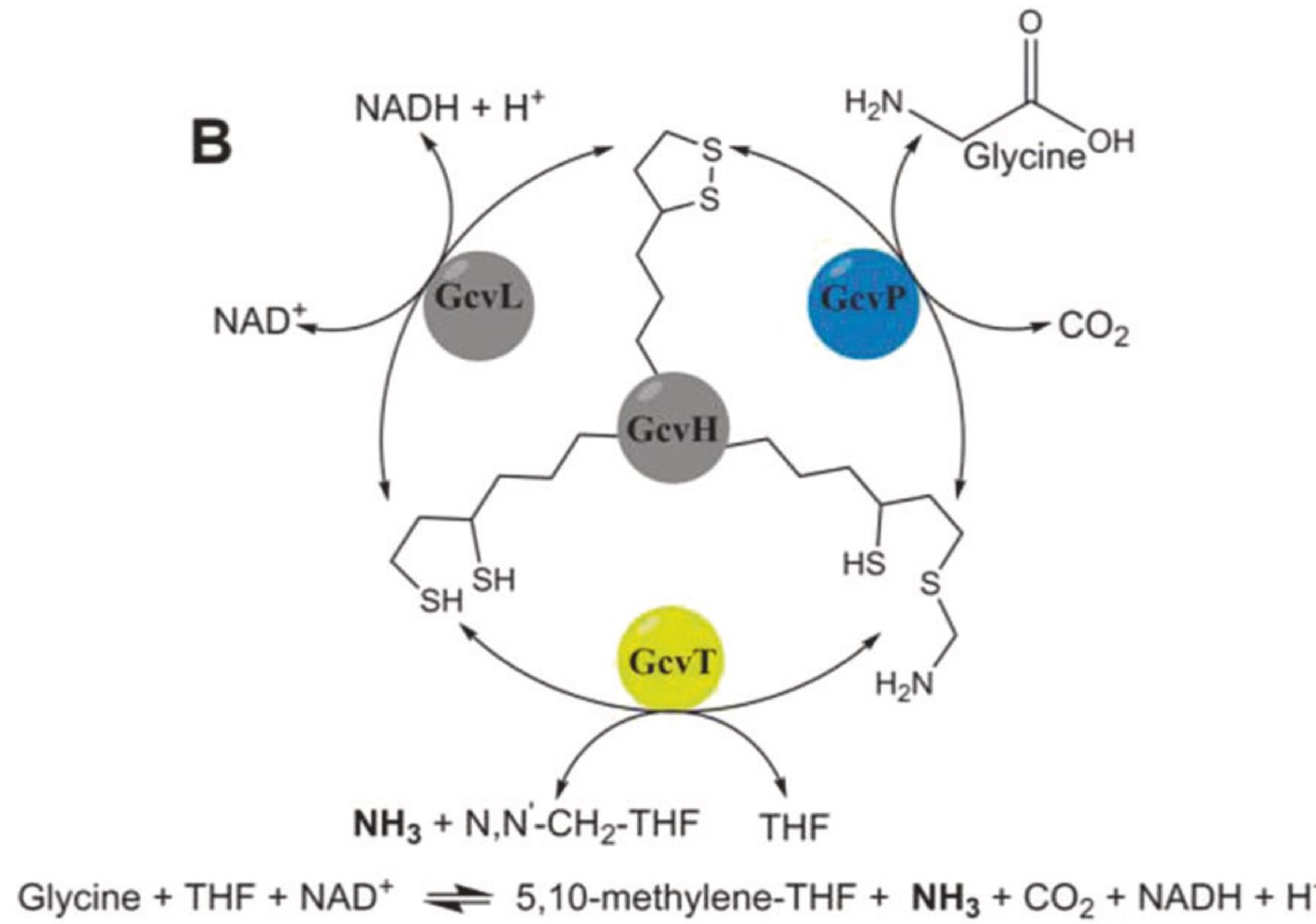
枯草芽孢杆菌

生物活性是由氨引起的。

③. Ammonia is derived from glycine cleavage

甘氨酸裂解系统（GCV）：三种酶（GcvL，GcvP，GcvT）和一种载体蛋白GcvH





在足够高的甘氨酸浓度下，所有测试的链霉菌都能产生一定水平的氨，从而抑制其他细菌的生长。

*S. coelicolor*和*S. griseus*，挥发性氨主要来自甘氨酸裂解（GCV）系统，GcvT是甘氨酸生产氨的关键酶。 gcvP的失活阻止*S. coelicolor*中氨气的生成。

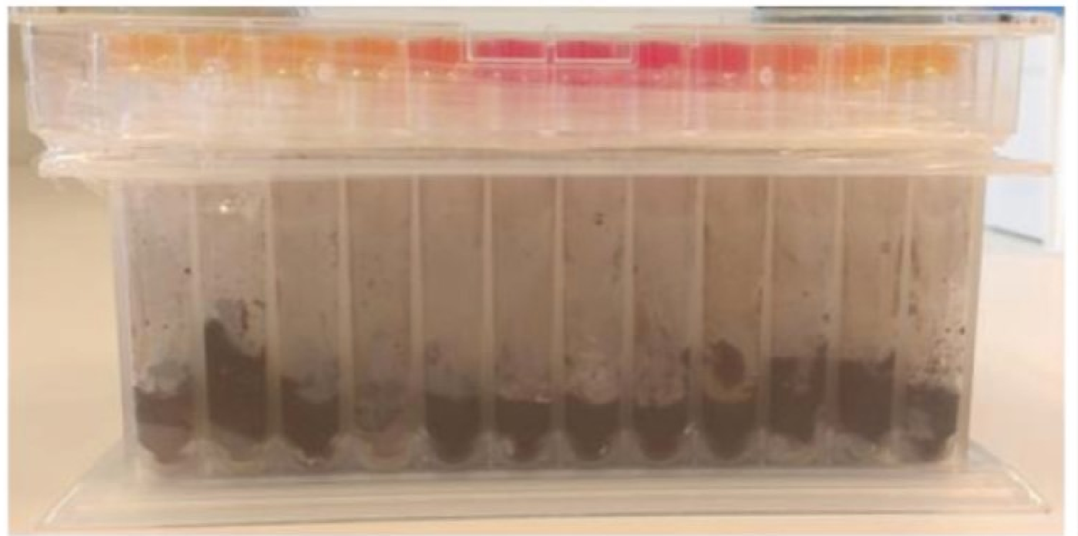
*S. griseus*中gcv基因的**沉默**（而非敲除），可能会出现基因残留表达。

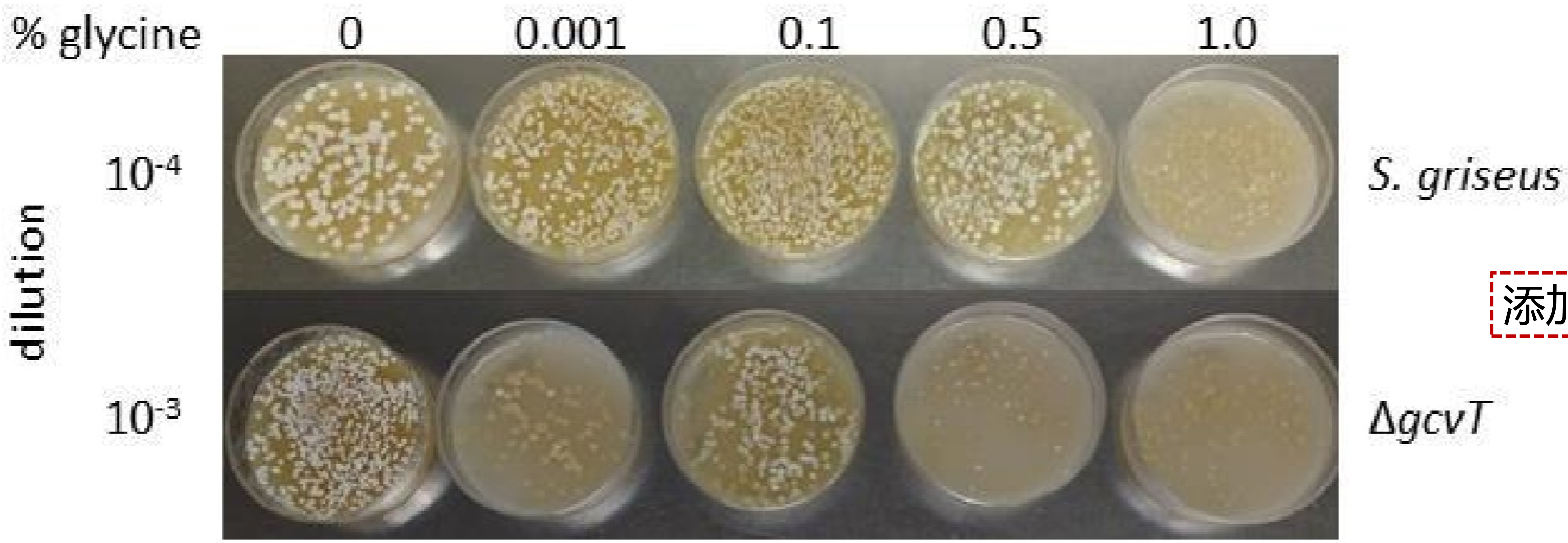
生物活性是由氨以甘氨酸裂解依赖性方式引起的。

④. Ammonia production in situ

在生态环境中是否也能发生远距离的挥发物介导的拮抗作用?

在土壤中加入*S.griseus*和gcvT突变体菌株，测定随着甘氨酸浓度增加，土壤顶空释放的挥发物的抗菌活性。

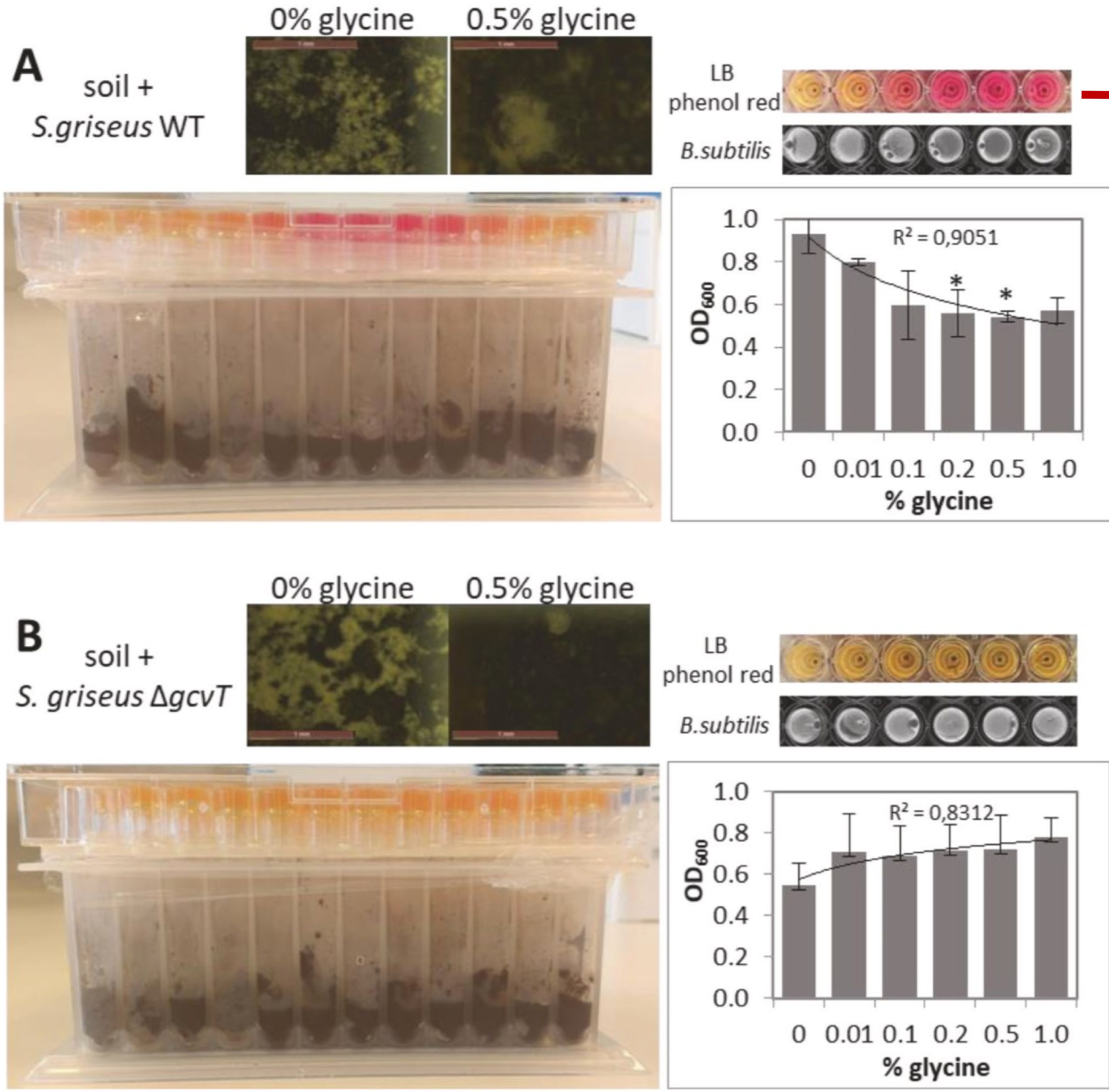




添加0.1%的甘氨酸生长最佳

采用稀释涂布法从不同甘氨酸浓度的土壤悬浮液中回收的链霉菌菌落。

④. Ammonia production in situ



氨气是由*S.griseus*产生的。

土壤中添加甘氨酸，*S.griseus*产生的挥发性氨对枯草芽孢杆菌的远距离抑制。

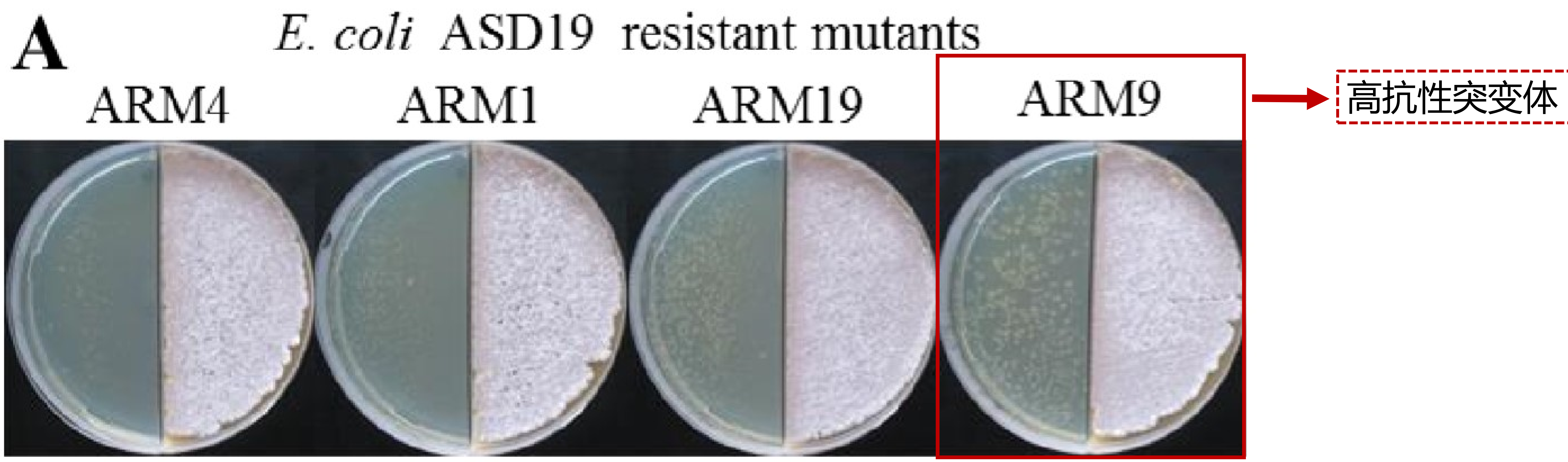
在自然环境中，甘氨酸是根系分泌物中的丰富氨基酸，使其在土壤中的存在成为可能（Bobille H *et al*, Zhalnina K *et al*）。

研究表明：

在甘氨酸存在的条件下，土壤环境中链霉菌产生氨，从而远距离杀灭其它细菌。

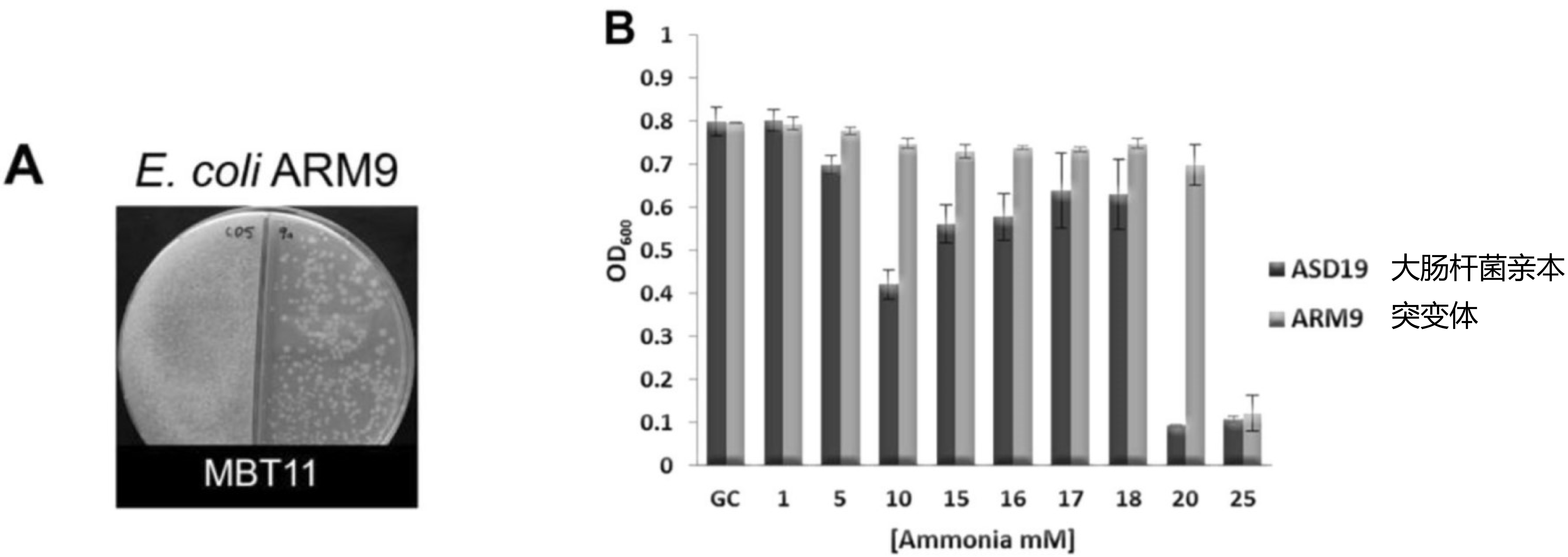
⑤. OmpR is key to ammonia resistance

为深入了解氨介导的抗微生物作用机制，选择**具有抗氨突变体的大肠杆菌**，来源于暴露在*Streptomyces* sp. MBT11. 产生的氨的环境中。



Colonies from *E. coli* strain ASD19 **resistant** to VCs produced by *Streptomyces* sp. MBT11.

⑤. OmpR is key to ammonia resistance



大肠杆菌对氨的敏感性临界点：20mM → 25mM ↑

⑤. OmpR is key to ammonia resistance

为了确定ARM9的突变性质，将其基因组序列与其亲本大肠杆菌ASD19的基因组序列进行比较。

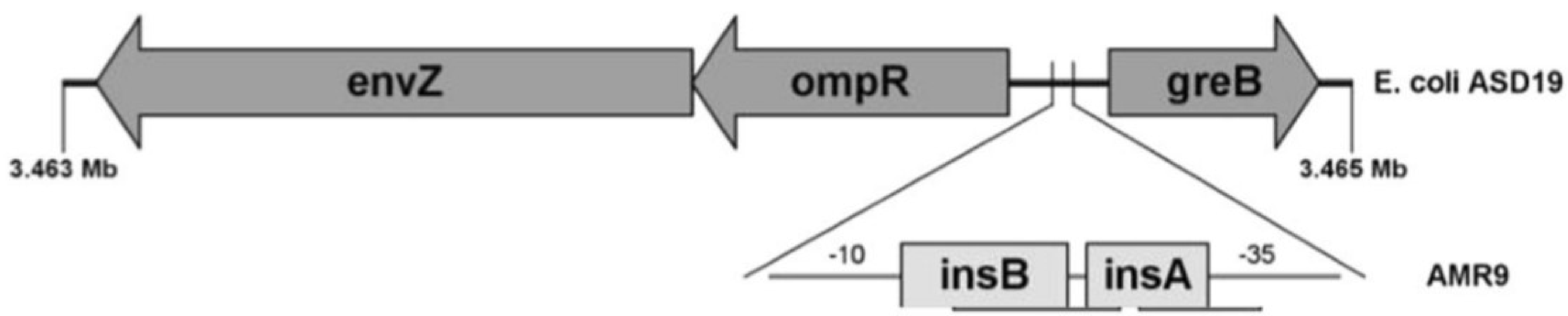
Feature ID	ARM9 vs ASD19 fold change	GeneID	Protein Product	UniProt/SwissProt Accession
			DOWN-REGULATED	
envZ	-16.43	947272	sensory histidine kinase in two-component regulatory system with OmpR	P0AEJ4
omrA	-15.86	2847746	N.A.	N.A.
yhdV	-15.86	947767	putative outer membrane protein	P64622
ompR	-14.84	947913	response regulator in two-component regulatorysystem with EnvZ	P0AA16
yrhB	-8.96	947948	stable heat shock chaperone	P46857
yfcV	-6.55	949109	putative fimbrial-like adhesin protein	P77288
dacD	-6.47	946518	D-alanyl-D-alanine carboxypeptidase,penicillin-binding protein 6b	P33013
yqhH	-6.20	946832	outer membrane lipoprotein, Lpp paralog	P65298
hlyE	-5.52	945745	hemolysin E	P77335
oweS	-5.52	947403	N.A.	P0AD35
phnE	-5.52	948625	N.A.	P16683
ycjO	-5.52	945888	putative sugar ABC transporter permease	P0AFR7
eutN	-5.17	946945	Ethanolamine catabolic microcompartment shellprotein	P0AEJ8
ydiM	-4.83	946196	putative MFS transporter, membrane protein	P76197
ygcG	-4.83	945247	TPM domain protein, putative phosphatase	P55140
yiaD	-4.54	948075	multicopy suppressor of bamB, outer membranelipoprotein	P37665
yafL	-4.48	944899	putative lipoprotein and C40 family peptidase	Q47151
yajR	-4.37	945058	putative transporter	P77726
hycA	-4.14	947193	regulator of the transcriptional regulator FhlA	P0AEV4
yzgL	-4.14	947933	N.A.	P76692
hofP	-3.96	947900	DNA catabolic protein	P45750

通过单核苷酸排列分析发现总共658个突变，其中198个引起了氨基酸的变化（插入或缺失）。

⑤. OmpR is key to ammonia resistance

在*ompR-envZ*启动子的-35和-10共有序列之间引入了两个插入元件（insA_31和insB_31），编码由反应调节因子OmpR和感觉激酶EnvZ组成的双组分调节系统（two-component system：TCS）。

TCS参与对环境信号的渗透调节，调节外膜孔蛋白OmpF和OmpC的表达。已知它们与渗透压和pH调节的抗生素耐药性有关，从而降低大肠杆菌细胞对VCs的反应性。

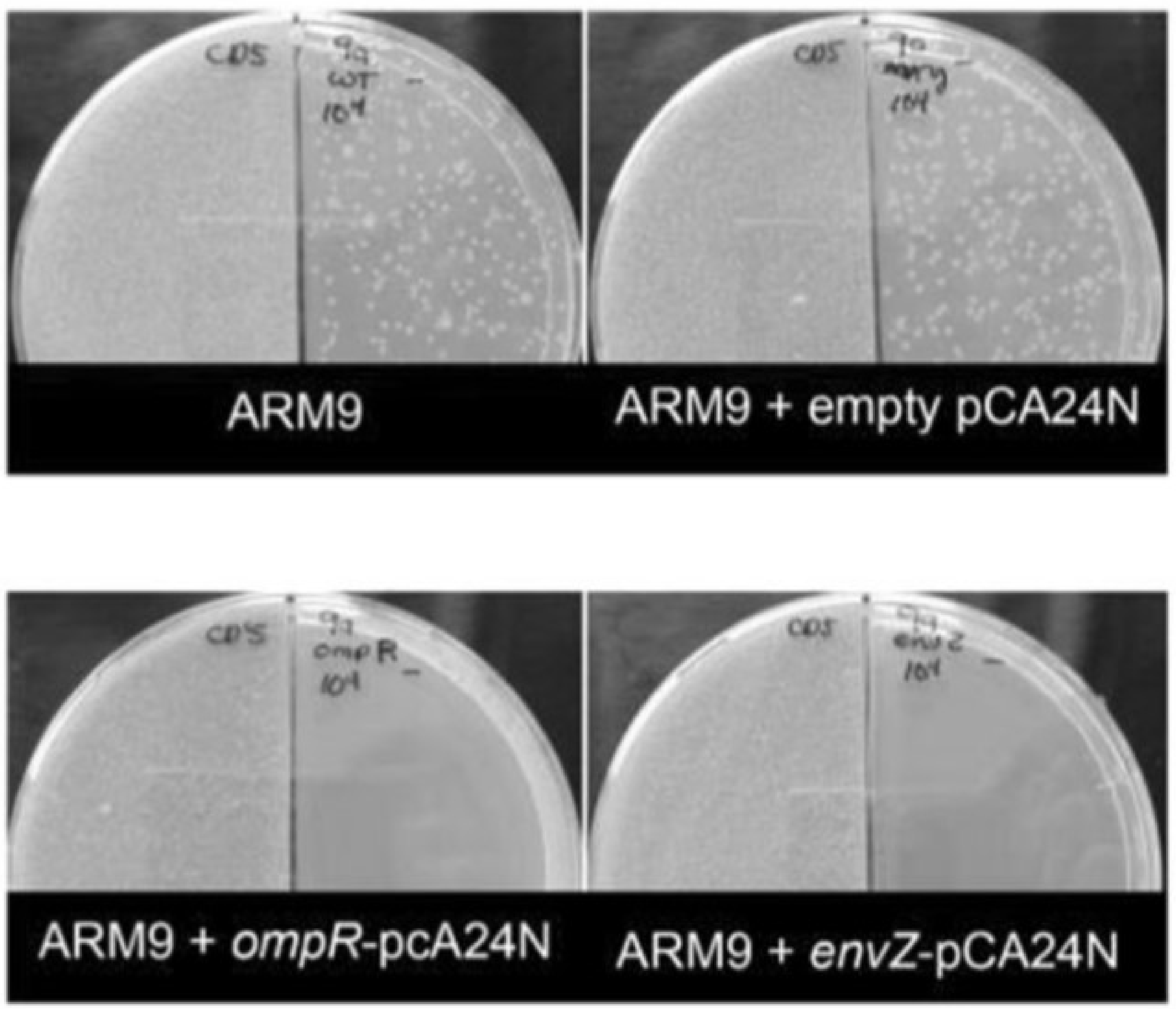


大肠杆菌*ompR-envZ*启动子中的插入序列决定了对氨的抗性。

⑥. Reduced transcription of the *ompR-envZ* operon is the cause of ammonia resistance

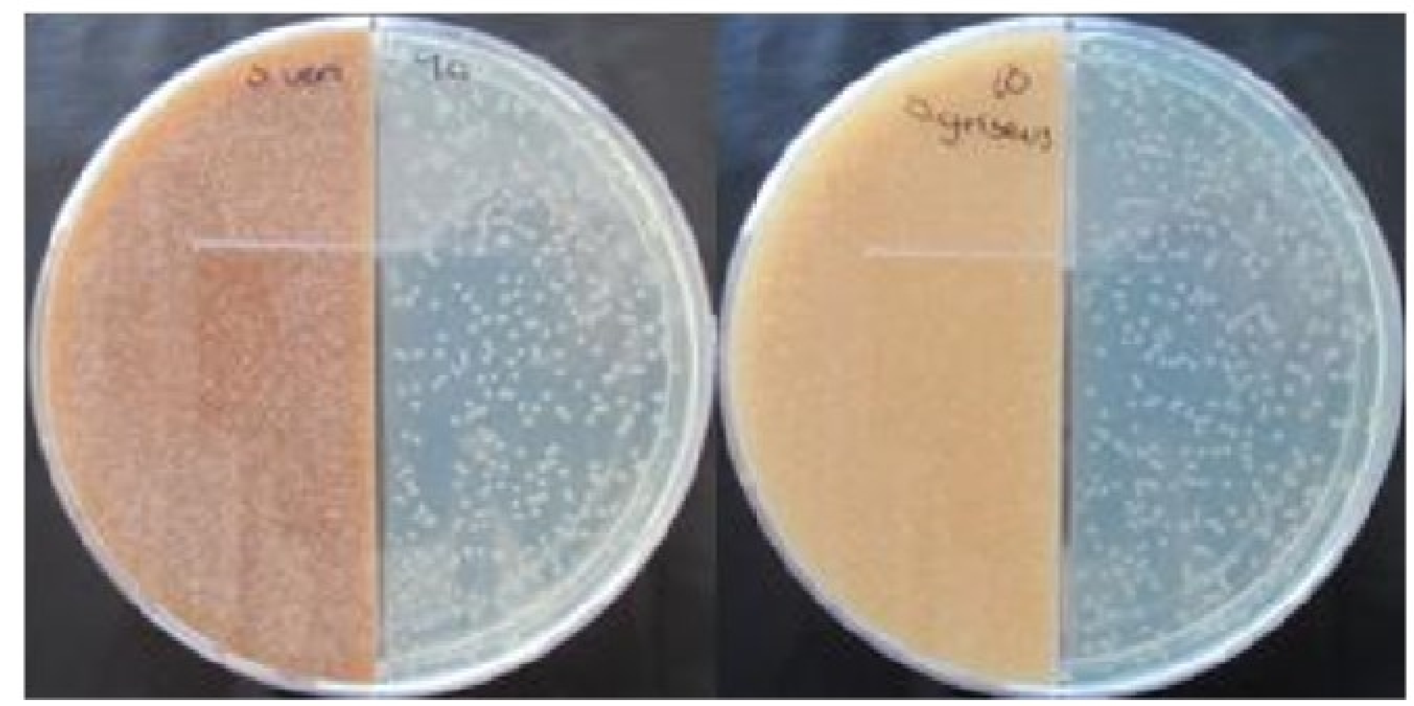
***ompR-envZ* 转录降低是获得氨抗性的主要原因?**

C



Growth of suppressor mutant ARM9 and transformants harboring either empty plasmid pCA24N, plasmid *ompR*-pCA24N (expressing *ompR*), or plasmid *envZ*-pCA24N (expressing *envZ*).

引入表达ompR或envZ的构建体可恢复氨的敏感性，而带有空质粒的转化体则继续具有抗性。



E. coli suppressor mutant **ARM9** is resistant against VCs produced by *S. venezuelae* and *S. griseus* grown at **pH 10** (buffered with glycine).

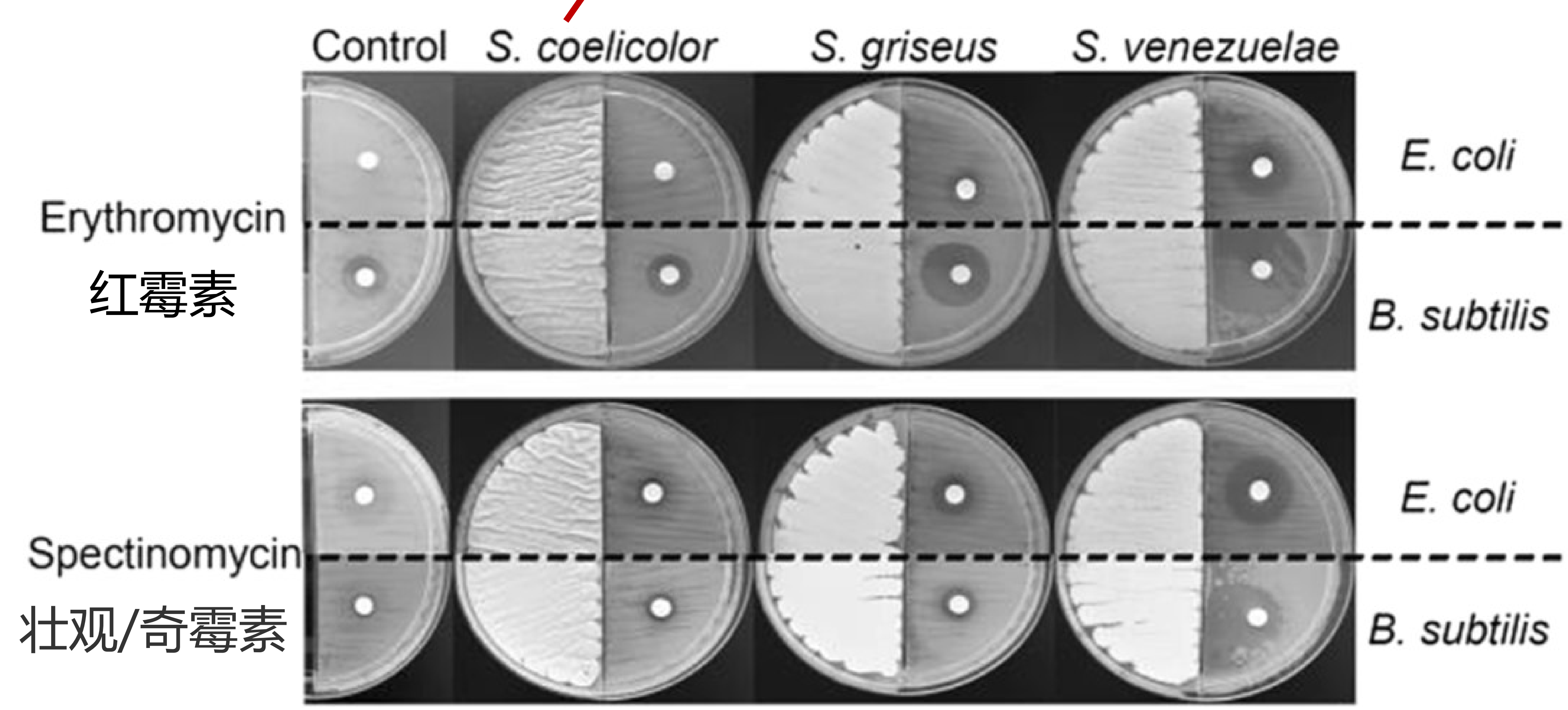
⑦. Ammonia released by Streptomyces modifies sensitivity to canonical antibiotics

链霉菌产生的挥发性化合物和可溶性抗生素之间的协同作用。

氨是具有挥发性的AMVC，可到达远距离处的菌落。因此假设该分子可能在与土壤中其他微生物的远距离竞争中发挥作用。 例如，改变由放线菌产生的其他抗生素的作用。

链霉菌接种4d后，接种*B. subtilis and E. coli* BREL606

Antibiotic	*E. coli*	*B. subtilis*
Ampicillin	NC	+
Erythromycin	+	+
Kanamycin	+	NA
Tylosin	NA	+
Actinomycin	NA	+
Spectinomycin	+	+
Streptomycin	NA	+



当产氨的链霉菌在受体细胞附近生长时，枯草芽孢杆菌和大肠杆菌对大多数抗生素的敏感性显着提高。

来自链霉菌的生物活性VCs在更长的距离上调节可溶性抗生素的活性，从而使它们能够利用其他细菌产生的抗生素，增强其自身VCs的活性。

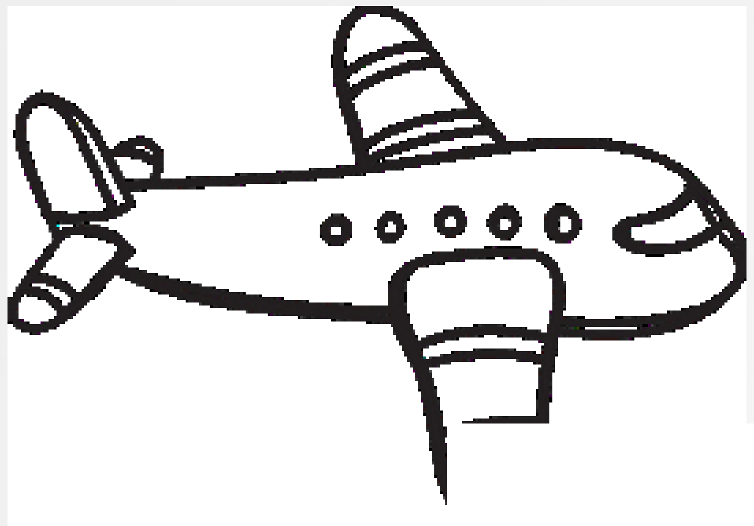
4. Discussion

Discussion

土壤中的链霉菌是自然界的药物制造者，目前一半以上的已知抗生素和许多其他具有生物活性的天然产物都由其生产。但是，这些细菌还会产生许多挥发性物质，这些分子会散布在土壤基质中，并可能远距离影响其他（微）生物。

甘氨酸是产生氨的前体，而甘氨酸裂解系统的失活使氨的生物合成失效。大肠杆菌细胞通过降低反应调节因子OmpR和感觉激酶EnvZ的表达，以在氨介导的抗菌作用中生存。氨被证明可以增强经典抗生素的活性，氨的释放可以帮助其他类型的次级代谢产物溶解和扩散，同时使竞争细菌敏感。

本研究首次表明土壤中生长的链霉菌可以产生大量具有挥发特性的氨，通过空气传播，从而远距离杀死细菌。链霉菌可以将氨作为廉价的空中武器来改变它们周围的环境，从而使自己的防御机制更加有效。抗微生物挥发性化合物（AMVC）领域的研究，为农业或医学应用提供新的机会，如用于作物保护和天然产物的发现。



Thank You !