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Bioorganic & Medicinal Chemistry 12 (2004) 3333–3338
Bioorganic & Medicinal Chemistry

Two novel antibiotics, Sch 419558 and Sch 419559, produced by *Pseudomonas fluorescens*: effect on activity by overexpression of RpoE

Shu-Wei Yang,* Ling Xu, Ronald Mierzwa, Ling He, Joseph Terracciano, Mahesh Patel, Vincent Gullo, Todd Black, Wenjun Zhao, Tze-Ming Chan and Min Chu*

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

Received 29 January 2004; accepted 23 March 2004
Available online 6 May 2004

Abstract—Two new secondary metabolites designated as Sch 419558 (**1**) and Sch 419559 (**2**), were isolated from the fermentation broth of *Pseudomonas fluorescens*. Structure elucidation of **1** and **2** was accomplished by spectroscopic data analyses including MS and NMR experiments. Both compounds were identified as lipopeptides containing valine and threonine linked with 1-amino-1-hydroxy-heptadec-9-en-2-one or 1-amino-1-hydroxy-pentadecan-2-one carbon chains, respectively. Characterization of the amino acids was further confirmed by amino acid analysis. Compounds **1** and **2** exhibited antibacterial activity against a sensitized *E. coli* strain with minimum inhibitory concentration of 0.3 and 0.6 μg/mL, respectively. Overexpression of RpoE in the *E. coli* strain increased the MIC over 60-fold for compounds **1** and **2**.

1. Introduction

The rapid development of multiple antibiotic-resistant pathogenic bacteria has been reported due to the intensive use of antibiotics during the past decade.[1] Bacterial strains resistant to all available antibacterial agents including vancomycin, which is considered as the last line of defense, have been recently identified among clinical isolates of certain bacterial species.[2] The discovery and development of effective antibacterial drugs with novel mechanisms of action have become an urgent task for infectious disease research programs. A genomic-based antibacterial approach was initiated to identify new targets by searching for genes essential to the growth of bacteria. The *rpoE* gene encodes a sigma-factor protein (σ^{24}). Specific sigma factors are required for transcription of subsets of genes.[3] Alternative sigma-factors, such as RNA polymerase sigma-E factor (RpoE), provide a means of transcriptional regulation in bacterial cells. RpoE is unique to bacteria and is essential for *E. coli* cell viability.[4] The expression of native RpoE in *E. coli* is naturally induced by perturbations to periplasmic protein folding caused by factors such as heat shock, overproduction of outer membrane proteins, or presence of misfolded periplasmic proteins prevalent in *dsb*, *surA*, *fkpA*, *htrM* mutant strains. Genes known to be regulated by RpoE encode proteins required for proper folding of secreted proteins;[5] these include *rpoH* (σ^{23}, the major heat shock sigma factor), *degP* (htrA, periplasmic protease), *rpoE*, *fkpA* (periplasmic peptidyl prolyl isomerase), and at least eight additional proteins identified by single dimension SDS-PAGE resulting from overexpression of RpoE. During the screening for inhibitors of RpoE with a large number of bioactive extracts from microbial sources, two novel metabolites Sch 419558 (**1**) and Sch 419559 (**2**) (Fig. 1), were discovered from the fermentation culture

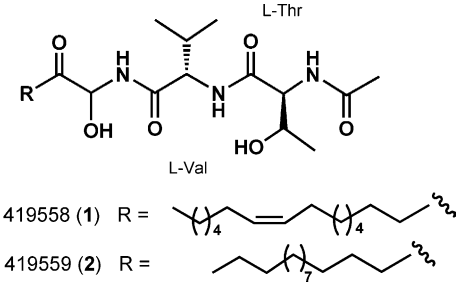

Figure 1. Structures of Sch 419558 (**1**) and Sch 419559 (**2**).

* Corresponding authors. Tel.: +1-908-740-7291; fax: +1-908-740-7152; e-mail: shu-wei.yang@spcorp.com

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doi:10.1016/j.bmc.2004.03.057

broth (culture number: Belt-2763) identified as *Pseudomonas fluorescens*. We now report on the fermentation, isolation, structure elucidation, and biological activity of these antibacterial compounds whose activity is affected by overexpression of RpoE.

2. Results and discussion

The crude EtOAc extract of fermentation culture broth was partitioned by a modified Kupchan method.[6] The bioactive CH_2Cl_2 fraction was separated on a polymeric CG-161 column followed by a size-exclusion LH-20 column chromatography. The active material was subjected to reversed-phase C18 HPLC purification to afford two pure components **1** and **2**. Their physicochemical properties are listed in Table 1.

The major component **1** was obtained as a white solid. Its molecular weight was determined to be 525 Da based on LC/MS (ESI+) data that showed the protonated molecular ion at m/z 526 $(M+H)^+$, as well as a strong sodium adduct ion peak at m/z 548 $(M+Na)^+$. The molecular formula was established as $C_{28}H_{51}N_3O_6$ by high-resolution FABMS (Calcd for $C_{28}H_{51}N_3O_6Na$: 548.3698. Found: 548.3703). The UV spectrum of **1** revealed strong end absorption at 220 nm with very weak absorption at 285 nm (see Table 1), which suggested the lack of a significant conjugated chromophore in the molecule. Absorptions at 3315 and 1663 cm^{-1} in the IR spectrum indicated the presence of hydroxyl and amide groups. The ^1H NMR spectrum of **1** revealed the presence of three amide NH doublets (δ 8.81, 9.10, and 9.98), and a large number of aliphatic signals (δ 1.09–1.22) suggesting a peptidic moiety and a long carbon chain unit in **1**. Two exchangeable singlets (δ 3.56 and 8.79) indicated the presence of two hydroxyl functionalities, which is consistent with the IR data. The ^{13}C NMR spectrum of **1** (Table 2) showed three amide carbonyls (δ 172.69, 171.77, and 170.49), two nitrogen attached methines (δ 59.53 and 59.16), one nitrogen–oxygen attached methine (δ 76.92), one oxygen attached methine (δ 67.67), which were consistent with the observation in ^1H NMR spectrum for peptidic moieties. Furthermore, two vinylic methine resonances with the same chemical shift (δ 130.21), as well as a total of 12 methylene carbon resonances (δ 22.90–38.37), were observed indicating the presence of unsaturation in the long aliphatic carbon chain portion of **1**.

Table 1. Physico-chemical properties and spectral data of Sch 419558 (**1**) and Sch 419559 (**2**)

	Sch 419558 (**1**)	Sch 419559 (**2**)
Melting point	180–182 °C (dec)	171–173 °C (dec)
ESI-MS (m/z)	526 $(M+H)^+$	500 $(M+H)^+$
	548 $(M+Na)^+$	522 $(M+Na)^+$
Molecular weight	525	499
Molecular formula	$C_{28}H_{51}N_3O_6$	$C_{26}H_{49}N_3O_6$
HR-FABMS (m/z)		
Calcd:	548.3698 $(M+Na)^+$	522.3519 $(M+Na)^+$
Found:	548.3703 $(M+Na)^+$	522.3528 $(M+Na)^+$
UV λ_{max} (nm)	220	220
	285 (very weak)	285 (very weak)

Table 2. ^1H and ^{13}C NMR spectral data of Sch 419558 (**1**)[a]

#	^1H (δ)	^{13}C (δ)
1	0.80 t, 7.0[b]	14.25 q[c]
2	1.09–1.22 m	22.90 t
3	1.09–1.22 m	31.98 t
4	1.09–1.22 m	30.00 t
5	1.09–1.22 m	30.00 t
6	2.00 m	27.51 t
7	5.40 m	130.21 d
8	5.40 m	130.21 d
9	2.00 m	27.51 t
10	1.09–1.22 m	29.65 t
11	1.09–1.22 m	29.46 t
12	1.09–1.22 m	29.40 t
13	1.09–1.22 m	29.19 t
14	1.60 m	23.88 t
15	2.66 m	38.37 t
16	—	207.59 s
17	6.31 d, 7.8	76.92 d
18	9.98 d, 8.4	—
19	—	172.69 s
20	5.05 dd, 3.0, 6.5	59.16 d
21	9.10 d, 8.4	—
22	—	171.77 s
23	5.40 m	59.53 d
24	8.81 d, 8.4	—
25	—	170.49 s
26	2.09 s	23.02 q
27	2.38 m	31.39 d
28	1.00 d, 6.8	19.72 q
29	1.02 d, 6.8	18.40 q
30	4.68 m	67.67 d
31	1.45 d, 6.4	20.01 q
17	3.56 s (OH)	—
30	8.79 s (OH)	—

[a] Recorded at 400 MHz for proton and 100 MHz for carbon spectra, respectively, in pyridine-d_5, δ = ppm.
[b] Coupling constants in Hz.
[c] Multiplicity was determined by APT and DEPT data.

Examination of 2D NMR spectral data from the HMQC–TOCSY experiment (1-bond ^1H–^{13}C correlation, see Fig. 1) suggested the presence of two amino acid residues (fragment A: valine, fragment B: threonine), a 1-amino-1-hydroxyl acetyl moiety (fragment C) and a single unsaturated 15-carbon chain residue (fragment D). The stereochemistry of the double bond (Δ 7,8) in fragment D was determined to be *cis*-configuration based on the observation of a very small coupling constant (~1 Hz) of H-7 and H-8. Since the unsaturated aliphatic chain contains 16 carbons if the carbonyl group is included, it becomes identical to palmitoleic acid carbon chain. Therefore, NMR data of palmitoleic acid are suitable for comparison purpose as a reference. The regiochemistry of the double bond as well as its configuration assignments on the 16-carbon chain for **1** were consistent with both proton and carbon NMR data of palmitoleic acid.[7] The connections of these four fragments were finally assembled on the basis of analysis of the HMBC experiment (long range ^1H–^{13}C correlation) as shown in Figure 3. HMBC correlations

of C-16 to H-14, H-15, H-17, and H-18 (NH) revealed that the amino-hydroxyl-ketone carbonyl at C-16 directly connected to the 15-carbon chain unit. Since this carbonyl group is connected to the aliphatic chain through a carbon–carbon bond, fragment C is not considered as an amino acid residue. Correlations of C-19 to H-17, H-18 (NH), H-20, and H-27 indicated that the valine was connected to the amino-hydroxyl-ketone, and correlations of C-22 to H-20, H-21 (NH), H-23, and H-30 further extended the connection of the valine to threonine. Finally, correlations of C-25 to H-23, H-24 (NH), and H-26 led to the assignment of the acetyl group at the N-terminus of threonine, allowing the complete structure assignment of **1**.

To confirm the composition of the peptidic portion and determine the stereochemistry (D or L-series) of amino acid residues of **1**, amino acid analysis was conducted. Two amino acids, threonine and valine, were observed after the hydrolysis with HCl. Although the threonine was linked to an acetyl group, the *N*-acetyl group was hydrolyzed under acidic condition. This result provided further support to the 2D NMR assignments. Furthermore, determination of the stereochemistry for threonine and valine was accomplished by a derivatization with *o*-phthalaldehyde (OPA).[8] As expected, the stereochemistry of both threonine and valine was found to be L-series, which is in agreement with most of naturally occurring amino acids (equivalent to the *S*-configuration designation for α-carbons in amino acids). Therefore, the two chiral centers at C-20 and C-23 were assigned to*S*-configuration, as shown Figure 1. The chiral center in the amino-hydroxy-ketone moiety (fragment C) at C-17, however, can not be determined by NMR methods in this study due to the lack of adjacent chiral center as a reference for comparison in NOESY experiments.

The molecular weight of **2** was determined to be 499 Da on the basis of analysis of LC/MS (ESI+) data that showed the protonated molecular ion at m/z 500 $(M+H)^+$, as well as a strong sodium adduct ion peak at m/z 522 $(M+Na)^+$. The molecular formula was established as C_{26} $H_{49}N_3O_6$ by high-resolution FABMS (Calcd for $C_{26}H_{49}N_3O_6$ Na: 522.3519. Found: 522.3528). The UV and IR spectra of **2** were identical to **1** indicating that **2** could be a congener of **1**. The ^1H and ^{13}C NMR spectral data of **2** (Table 3) provided supportive evidence to show very similar patterns of proton and carbon signals in comparison with **1**. The difference appeared to be the absence of two vinylic carbons in **2** based on analysis of the ^1H and ^{13}C NMR spectra. The HMQC–TOCSY data not only confirmed the presence of fragments A, B, and C, which are the same as in **1**, but also indicated the presence of a 13-carbon chain unit assigned as fragment E, shown in Figure 2. Analyses of HMBC data (Fig. 3) permitted the assignments for all fragment connections. The stereochemistry of two chiral centers at C-18 and C-21 in **2** was assigned to be the same as those in **1**.

The functionality of *N*-(1-hydroxy-2-oxo-alkyl)-amide (fragment C) is very rare but occurs in natural products, such as cyclotryprostatin D and pseurotin-F1 and F2

Table 3. ^1H and ^{13}C NMR spectral data of Sch 419559 (**2**)[a]

#	^1H (δ)	^{13}C (δ)
1	0.88 t, 7.0[b]	14.64 q[c]
2	1.34 m	23.30 t
3	1.21 m	32.49 t
4	1.19–1.36 m	29.86 t
5	1.19–1.36 m	29.97 t
6	1.19–1.36 m	30.14 t
7	1.19–1.36 m	30.27 t
8	1.19–1.36 m	30.40 t
9	1.19–1.36 m	30.36 t
10	1.19–1.36 m	30.55 t
11	1.19–1.36 m	30.30 t
12	1.69 m	24.27 t
13	2.93 m	38.73 t
14	—	208.01 s
15	6.28 br d, 7.0	77.33 d
16	10.02 d, 8.0	—
17	—	173.06 s
18	5.08 dd, 3.0, 6.5	59.56 d
19	9.13 d, 8.0	—
20	—	172.15 s
21	5.31 dd, 3.0, 6.5	59.93 d
22	8.55 d, 8.0	—
23	—	170.85 s
24	2.16 s	23.38 q
25	2.46 m	31.74 d
26	1.07 d, 7.0	20.09 q
27	1.09 d, 7.0	18.77 q
28	4.75 m	68.06 d
29	1.53 d, 7.0	20.39 q
17	8.79 br s (OH)	—

[a] Recorded at 400 MHz for proton and 100 MHz for carbon spectra, respectively, in pyridine-d_5, δ = ppm.
[b] Coupling constants in Hz.
[c] Multiplicity was determined by APT and DEPT data.

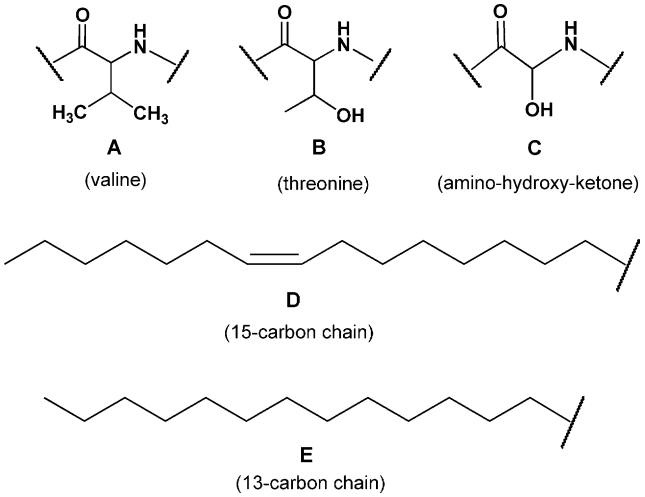

Figure 2. HMQC–TOCSY data of Sch 419558 (**1**) and Sch 419559 (**2**).

isolated from *Aspergillus fumigatus*,[9,10] and *N*-acetyl-α-hydroxy-β-oxotryptamine from *Streptomyces ramulosus*.[11] The structures of compounds **1** and **2** represented unique di-amino peptide linked with a long-chain lipid through unusual functionality of *N*-(1-hydroxy-2-oxo-alkyl)-amide.

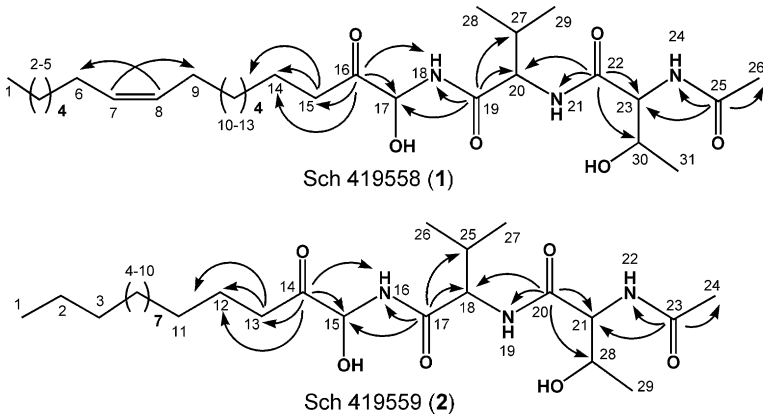

Figure 3. HMBC data of Sch 419558 (**1**) and Sch 419559 (**2**).

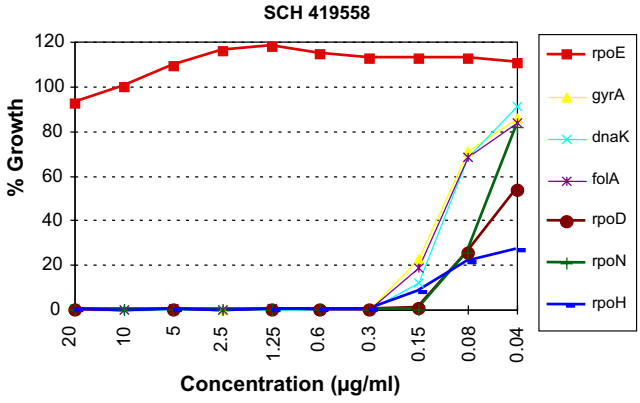

Figure 4. Dose–response of Sch 419558 (**1**) for growth inhibition of *E. coli* strain (HS294) containing overexpression plasmids for various target proteins, including the major σ-factors for *E. coli*.

Compounds **1** and **2** exhibited antibacterial activity against a sensitized *E. coli* strain (HS294) with MIC = 0.3 and 0.6 µg/mL, respectively. The sensitized *E. coli* strain (HS294) is a genetically engineered strain of the wild-type *E. coli* with, for instance, lesion in *lpxC*, the deletion of the two genes (*acrAB* and *emrAB*) associated with efflux pumps and the insertion of the resistance markers for gentamicin, erythromycin, and β-lactams. Compound **1** was also active against *Staphylococcus aureus* (strain RN4220) at 40 ug/mL while compound **2** had an MIC >40 ug/mL for *S. aureus*. As shown in Figure 4, the major component **1** displayed >60-fold increase in MIC when RpoE is overexpressed. In preliminary studies, component **2** also showed a higher MIC when RpoE was overexpressed, however, there was insufficient material available to perform a confirmation and thorough evaluation on the impact of RpoE overexpression on the antibacterial activity of component **2**.

3. Conclusions

Two novel antibacterial agents **1** and **2** were produced by *P. fluorescens*, which is a bacterial contaminant from the original fungal microorganism. Compounds **1** and **2** contain the same amino acid residues, but different aliphatic chain attachments. Both lipopeptides lost their antibacterial activity significantly against sensitized *E. coli* when RpoE was overexpressed, which indicated that the antibacterial activity of **1** attributed to the inhibition of RpoE or the activity against proteins that are overexpressed when RpoE is overexpressed.[5] This discovery also suggested that RpoE inhibitors have potential to become clinical candidates for novel antibacterial agents. To the best of our knowledge, this is the first reported case of the MIC enhancement of lipopeptide antibiotics by overexpression of RNA polymerase sigma-factor protein (RpoE).

4. Experimental

4.1. General procedure

Melting points were recorded on a MEL-TEMP apparatus (Laboratory Devices, Cambridge, MA) and are uncorrected. IR and UV spectra were obtained using a Nicolet FTIR model 10-MX and a Hewlett-Packard '8050 A' ultraviolet–vis spectrophotometer, respectively. LC–MS analyses were performed on a triple-stage quadrupole TSQ-7000 mass spectrometer equipped with ESI (electrospray ionization)/APCI (atmospheric pressure chemical ionization) interfaces (Finnigan MAT, San Jose, CA, USA) that linked to a Hewlett-Packard HP-1090 Series X system. HR-FABMS data were produced by a VG ZAB-SE mass spectrometer in a glycerol–thioglycerol matrix. Both ^{1}H and ^{13}C NMR spectra were recorded using Varian XL-400 instruments operating at 400 and 100 MHz, respectively.

4.2. Microorganism

The producing microorganism was isolated from a soil sample collected in forest area of Washington State near Seattle. The producing culture was originally collected as a fungal microorganism with bacterial contamination. The bacterial strain, as a contaminant, was later found to be responsible for the RNA polymerase sigma-

E factor (RpoE) activity, and it was isolated from the original fungus. The producing bacterium was further characterized and determined as *Pseudomonas fluorescens* on the basis of taxonomic analyses, including colony morphology and VITEK biochemical studies.[12]

4.3. Fermentation

The frozen whole broth of the producing organism (1 mL) was transferred to a germination medium consisting of BBL Trypticase soy broth 30 g/L. The culture was incubated at 30 °C for 48 h on a rotary shaker at 250 rpm. Approximately 1% inoculum of this culture was transferred to fermentation medium consisting of (g/L): Traders pharmamedia, 20; cerelose, 20; and $CaCO_3$, 4. The culture was fermented at 30 °C for 48 h on a rotary shaker at 250 rpm to produce the RpoE active components.

4.4. Isolation

The fermentation broth (90 L) was extracted with EtOAc. The crude extract was introduced to a modified Kupchan liquid partition method[6] as following: The extract was dissolved in MeOH–H_2O (9:1) and partitioned with equal amount of hexane. After separation, the aqueous MeOH portion (lower phase) was adjusted to 20% H_2O and partitioned with an equal amount of CCl_4. The aqueous MeOH phase (upper phase) was separated, then adjusted to 40% H_2O and partitioned with an equal amount of CH_2Cl_2.[13] The RpoE inhibitory activity was detected in both CCl_4 and CH_2Cl_2 layers. The combined active sample was introduced to Amberchrom CG 161 resin (Toso Hass), and eluted with a MeOH–H_2O step gradient. The 90% MeOH fraction was found to be active in the RpoE assay, and further chromatographed on Sephadex LH-20 column using CH_2Cl_2–MeOH (7:3) as a mobile phase. The enriched active complex was purified by reversed-phase HPLC (YMC C-18 semi-preparative column 250×20 mm with a guard column 50×20 mm, S-5, 120 Å, 85% aqueous-MeOH isocratic elution, 15 mL/min, UV = 220 nm) to obtain the major component **1** (6 mg) and the minor component **2** (3 mg).

4.5. Biological assay

E. coli strain HS294 (W3110 Δ*acrAB* Δ*emrAB lpxC** Δ*ompT lacZ*:: (GmR::T7RNAP::*ampC*P99::*lacIQ*::EmR) and other uncharacterized mutations) was transformed with various genes (*rpoD*, *rpoE*, *rpoH*, *rpoN*, *gyrA*, *dnaK*, and *folA*) that had been cloned into the expression vector pET29 (Novagen, Madison, WI). Cultures of each transformed strain were grown overnight at 30 °C in Terrific Broth (TB). A 1:10 dilution of the overnight culture was made into fresh TB media containing 0.4 mM IPTG and grown for ~2 h. The cultures were adjusted to OD 540 nm = 0.1 in sterile saline (~ 1× 107 CFU/mL). A 1:400 dilution of the saline suspension was made into TB media containing 0.4 mM IPTG. For growth inhibition experiments, 100 μL of the inoculum is added to each well of a 96-well microtiter plate containing the appropriate dilutions of antibacterial compounds. In the preliminary screening, culture concentration 0.4 X[14] in DMSO and further dilutions (0.2×, 0.1×, 0.05×, and 0.025×) were screened. For activity monitoring during fractionation, concentrations at 10 μg/mL and at further dilutions (5, 2.5, 1.25, and 0.63 μg/mL) were assayed. The plates were incubated for 18 h at 37 °C. Percentage of growth was determined by measuring the OD_{540} for each well and comparing to growth and no-growth control wells.

Acknowledgements

Authors wish to acknowledge Mr. P. Bartner for high resolution FABMS data, Mr. P. Cai for optical rotation data, Ms. C. Cramer for taxonomy information, and Dr. F. Perini from Nebraska Medical Center for amino acid analysis and D/L determination of the compounds.

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12. VITEK biochemical study was performed by Bio-Merieux Vitek Laboratory. This taxonomic analysis is considered a standard methodology for characterization

and identification of microorganisms on the basis of carbon and nitrogen uptake pattern recognition during the growth in a wide variety of nutrition media and comparison with microorganisms in the database.

13. The method was modified by using CH_2Cl_2 to replace CCl_4 due to the environmental concern. The most of RpoE activity was found in the second CH_2Cl_2 partition.
14. 'X' here referred to the original culture concentration.