Enzymatic activities, herbicide resistance and plasmid characteristics of the indigenous *Agrobacterium tumefaciens* "*SDB 0012*"

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ABSTRACT

An indigenous Agrobacterium tumefaciens strain "SDB0012" was identified at the National Institute for Promotion of Horticultural Export-University of Gezira, since 2005 and was subjected to different biological and molecular tests. These included expression of enzymes, production of acids from carbohydrates, resistance to different herbicides, sensitivity to antibiotics, type of tumors and plasmid(s) characteristics. Qualitative tests showed positive results for oxidase, urease, and catalase enzymes. This strain successfully utilized fructose, lactose, raffinose, sucrose and manitol as sources of carbon. Whereas, other carbohydrates such as starch, glucose, galactose and maltose were not utilized. It showed resistance against glyphosate (the active ingredient of roundup) and 2,4-D herbicides and sensitivity to pendimethalin (the active ingredient of stomp). The tumor caused by this strain showed shoots like growth on the surface of potato discs, which indicates the presence of nopaline-type plasmid(s). This strain showed resistance against penicillin (30mg), chloramphenicol (30mg) and ciprofloxacin (30mg). Therefore, it was described as a multi-resistant or a superbug bacterium. Results indicated the presence of two distinguished plasmids of close molecular weights ranging between 2.0 and 2.4 kb, while molecular weight of the indigenous isolate of Escherichia coli was estimated to be 2.0kb. The presence of the two plasmids in the bacterial cell of SDB0012 might explain the presence of more than one antibiotic Agrobacterium tumefaciens, glyphosate resistance, antibiotic resistance, tumors, plasmids, superbug bacterium, enzymatic activity resistance gene.

Key words: *Nopaline, antibiotic, superbug, teratoma, plasmid, tumor.*

INTRODUCTION

Agrobacterium tumefaciens is an alphaproteo-bacterium of the family *Rhizobiaceae*, which includes the nitrogen fixing legume symbionts; it is a rod shaped and Gram negative bacteria (Intrieri and Buiatti, 2001). Unlike the nitrogen fixing symbionts, tumor producing *Agrobacterium* is parasitic (Hooykaas *et al*., 1988). It is a soil bacterium that infects wound sites on a wide range of plant species and induces the development of crown gall tumors or hairy roots. These growth responses are resulted from a natural genetic engineering event, in which a specific region of DNA from Ti (tumor-inducing) or Ri (root-inducing) plasmid is transferred from *Agrobacterium* to plant cell and incorporated at a semi-random

location into the plant genome (Zupan *et al.*, 2000). The wide variety of plants affected by *Agrobacterium* makes it of great concern to the agriculture industry (Moore *et al.*, 1997). It infects over 140 plant species; the hosts for *Agrobacterium* are mostly dicotyledonous plants, but also include a few gymnosperms and several monocotyledonous plants (De Cleene and Ley, 1981). The strain specificity for plant genotypes and tissue types has been well documented. The DNA transmission capabilities of *Agrobacterium* have been extensively exploited in biotechnology as a means of inserting foreign genes into plants (Schell and Van Montagu,1977 and Zambryski *et al.*, 1983). Moreover, *Agrobacterium* provides a good tool of selection of resistant plants in breeding programs using the technique of agro-inoculation, as it has the ability to transfer cloned tandem repeats of the viral DNA into host plants On the other hand, *Agrobacterium* plays an important role in identification of the natural anticancer compounds to be used in treatment of cancer in human beings (Grimsley *et al.*, 1987).

In contrast to the linear chromosomes found in eukaryotic cells, bacteria were found to have single, covalently closed, circular chromosomes (Charlebois, 1999). The first convincing evidence that some bacteria have multiple chromosomes came from studies on *Rhodobacter sphaeroides* (Suwanto and Kaplan, 1989 and 1992). In general chromosomes carry genes that are essential for growth of the organism under all conditions. In contrast, plasmids carry genes that are not needed under all growth conditions; they are dispensable under certain growth conditions; for example, if a plasmid encodes antibiotic resistance it may be needed when cells are exposed to that antibiotic but not needed when cells are growing in the absence of the antibiotic. The most commonly used antibiotics in genetic engineering are ampicillin, kanamycin, tetracycline and chloramphenicol (Henryk *et al.*, 2003). On the other hand, Ti plasmid classification is based upon the general type of opine catabolism coded for by the plasmid *e.g.* octopine and nopaline-type plasmids code for the synthesis and anabolism of octopine and nopaline, respectively (Binns *et al.*, 1998).

Recently, a priority was given to isolate and identify indigenous *Agrobacterium* strain that could enhance biotechnology applications in Sudan (Fuad, 2007). These efforts succeeded in identification of the indigenous isolate of *A. tumefaciens* "SDB0012". The main objective of this study is to explore some of the valuable characteristics of this strain and its prospects to be used as a vector in gene transformation.

MATERIALS AND METHODS

The *Agrobacterium tumefaciens* isolate "SDB0012" was subjected to different biological and molecular tests; these included expression of enzymes, production of acids from carbohydrates, resistance to different herbicides in addition to its sensitivity to antibiotics, type of tumors and plasmid(s) characteristics. For preparation of *Agrobacterium tumefaciens* for the different experiments; the growth medium Yeast Manitol Broth (YMB) in each flask was inoculated with one colony of the bacterial isolate. The flasks were placed on a shaker at a speed of 150 strokes per minute for 48 hours at 28^0C.

Potato discs bioassay

For preparation of potato discs; the ends of potato tubers were cut away and cylinders cut by the cork borer into small discs and placed (5 discs per Petri dish) in bleach by gently pushing the discs into the agar. One drop (0.05ml) of the prepared inoculum was added as a treatment on the tops of each disc. The edge of each Petri dish was sealed with parafilm strips to prevent moisture loss during

the incubation period. The Petri dishes were kept in dark at 28^0C in a horizontal position to keep the inoculum on the top of the discs. Emerged tumors were counted after 21 days. Potato discs used as control were treated with sterilized distilled water.

Enzyme activities and digestion of carbohydrates

Testing for oxidase, urease and catalase and production of acids from carbohydrates were preformed according to procedures given by Chikara *et al.* (2000), Collee *et al.* (1996), Collins *et al.* (1995) and Serfontein and Staphorst (1994), respectively. The tested carbohydrates included glucose, galactose, fructose, sucrose, maltose, lactose, raffinose, starch and mannitol.

Response of the indigenous *Agrobacterium* to application of herbicides

Experiments were conducted to examine response of the indigenous strain of *Agrobacterium tumefaciens* "SDB0012" to the application of glyphosate, 2,4-D and pendimethalin. Glyphosate and 2,4-D were applied at dilution rates of 1:200, 1:1, 1:2 and 1:3 of the herbicide: distilled H_2O, while pendimethalin was tested at a dilution rate of 1:200 only. For the preparation of Nutrient Agar Selective *Agrobacterium* medium; 28 gm nutrient agar and 20 g sucrose were dissolved in one liter of distilled water at boiling temperature, autoclaved at 121°c for 15 min. One g of chloramphinicol was added after the medium was cooled at room temperature.

One ml of the bacterium was added to NASA medium in Petri dishes at room temperature and left to consolidate. Three holes of one cm in diameter and one cm depth were made in the solid medium then the three herbicides were added at the different concentrations in separate Petri dishes in a completely randomized design (CRD) with three replications. Each concentration was represented by two Petri dishes in each replication. Five *ul* of each herbicide were applied to a separate hole. Six tubes filled with one ml distilled H_2O were used as control. To test the effect of incubation period on herbicide resistance, the three herbicides were applied at a rate of 1 herbicide :200 distilled water to NASA medium containing *Agrobacterium* and incubated for 2, 7 and 15 days. This experiment was conducted in a CRD with three replications and each treatment was represented by two Petri dishes in each replication.

Type and number of tumors

Type of tumors was visually identified according to presence or absence of shoot like structures on the tumor surface. Number of tumors grown on each potato disc was recorded.

Sensitivity to antibiotics

Sensitivity to the antibiotics was performed according to the procedure given by Collee *et al.,* 1996). The antibiotics used were chloramphenicol (30mcg), gentamycin (10mcg), pencilin (30mcg), tetracyclin (30mcg), pefloxicin (30mcg) and ceprofloxacin (30mcg). To test for number of plasmids contained in the bacterium cell; extracted DNA of *Agrobacterium tumefaciens* "SDB 0012" and a local isolate of *Escherichia coli* were used in addition to a ladder DNA kindly provided by the National Institute of Cancer Research. This experiment was done twice at the Molecular Laboratory of the National Institute of Cancer Research- University of Gezira, Sudan. The plasmid DNA isolation of *Agrobacterium* and *E. coli* was conducted according to the alkaline-detergent method reported by Dillon *et al.* (1985). The extracted plasmid DNA was electrophoresized in 0.70% agrose gel. The gel was prepared by melting 0.7 g of agarose (Sigma) in 100 ml of 1xTBE buffer (prepared by diluting a 10 x TBE buffer consisting of

890 mM Tris, 890 mM Boric acid and 25 mM EDTA-PH 8.3). Five ml of ethidium bromide were added. Prior to casting the gel. the comb was adjusted and the gel was poured (making sure that there were no bubbles). While the gel was solidifying, DNA sample mixtures were prepared for electrophoresis as follows: for each sample, approximately 5ul of DNA were added to 1 ul of a buffer containing 30% sucrose and 0.01% bromophenol blue. After the agrose gel had solidified, the comb was removed with gentle back and forth motion and the gel was then immersed in 1 x TBE buffer. The buffer was added until it reached a level of ~3-5 mm above the gel surface; the sample mixtures were loaded into wells using a plastic-tipped micro-pipette .The apparatus (Amersham Pharmacia Biotech) was closed and the power was turned on (ESP 301 power supply). The voltage was adjusted to 100 v (200mA) and the running was continued without cooling for 2 hrs.

Table (1) shows the results of visual characterization, enzymatic activities and assimilation of carbohydrates by the indigenous strain SDB0012.

Oxidase test

A deep purple colour on the reagent paper "oxidase disk" that contained phenylene-diamine-p-dihydrochloride was observed after addition of the bacterial suspension, indicating a positive test for the presence of oxidase enzyme (Table 1). This change in color was resulted from oxidation of phenylene-diamine-p-dihydrochloride by the oxidase enzyme. Cytochrome oxidase is an enzyme found in some bacteria that transfer electrons to oxygen, the final electron acceptor in some electron transport chains (Xianggan *et al.*, 2003). It was concluded that the indigenous bacterium contained the gene(s) for oxidase in its genome and it was considered to be one of the characteristics of this bacterium. Oxidase enzyme was found in bacteria belonged to the species *Neisseria, Pseudomonas* and *Vibro* (Xianggan *et al.*, 2003). In general, oxidase enzyme has an herbicide resistance nature whether it is obtained from bacteria or plant. Xianggan *et al.* (2003) stated that oxidase enzyme of *Agrobacterium tumefaciens* was used to develop herbicide resistance crops in maize and Soya bean. Moreover, oxidase obtained from plants is used as a selectable marker gene in different crops for *Agrobacterium tumefaciens*- mediated transformation. They also reported the isolation of plant protoporphyrinogen oxidase (PPO) genes and the isolation of herbicide-tolerant mutants. Subsequently, an Arabidopsis double mutant (Y426M + S305L) was used by Xianggan *et al.* (2003) to develop a selectable marker system for *Agrobacterium tumefaciens*-mediated transformation of maize (*Zea mays*) and to obtain multiple events tolerant to the PPO family of herbicides.

Urease test

Urease enzyme activity is an important character in differentiating Enterobacteria. If the strain is urease-producer, the enzyme will breaks down the urea by hydrolysis to give ammonia and carbon dioxide (Chikara *et al.*, 2000). Similarly, a positive result was obtained in this study after addition of urea (46%N), as the colour of the indicator changed to pink (Table 1). Therefore, expression of urease enzyme was considered to be one of the characteristics of the indigenous isolate. Urease has many applications in removal of urea from surroundings. For example, Chikara *et al.* (2000) detected urease activity in the hemolymph of the silkworm, *Bombyx mori* from the beginning of spinning to the pharate adult stage if the larvae were reared on mulberry leaves. These results suggest that the silkworm larvae acquire the host plant urease specifically at the end of the feeding stage in

order to degrade urea accumulated in the hemolymph.

Catalase test

Oxygen bubbles were obviously observed after addition of the bacterial culture to hydrogen peroxide. This result indicated the presence of the catalase enzyme, which broke down hydrogen peroxide to oxygen and water (Table 1). Examples of catalase producing bacteria are all species of genus *Staphylococcus* and those of the *family Enterobacteriaceae,* which includes *Citrobacter, E. coli, Enterobacter, Klebsiella, Shigella, yersinia, Proteus, Salmonell*a and *Serratia.*

Table (1): General characteristics of the indigenous strain of Agrobacterium tumefaciens "SDB 0012".

Test	Characteristics
	Physical characteristics
Colony characteristics	Colour: whitish. Elevation: convex. Margin: smooth and round. Transparency: obique.
Motility test	diffuse hazy growth.
	Cultural and biochemical characteristics
Aerobiosis	Micro-Europhilic (bacterial growth on the bottom of the tube).
Oxidase test	*+ve.*
Urease test	*+ve.*
Catalase test	*+ve.*
Indole test	*-ve* (absence of tryptophanase).
Production of acids from carbohydrates	*+ve*: fructose, lactose, raffinose, mannitol. *-ve*: starch, glucose, galactose, maltose.
Production of H_2S	*-ve* (lack of sulfur containing amino acids).
Antibiotic resistance	*+ve*: penicillin, chloramphenicol, ceproflaxacin. *-ve*: gentamycin, oflaxacin, tetracycline.
Herbicide resistance	glyphosate (+), pendimethalin '2-4-D' (+), stomp (-).
Type of tumors	Nopaline type (teratoma-like tumors =shoot like growth on the surface of potato discs).
Number of plasmids	Two plasmids (superbug bacteria).
	Molecular characterization
Number of plasmids	2 plasmid with similar size

Table (2): Response of Agrobacterium strain to application of herbicides at different dilution rates.

Dilution rate (herbicide: distilled water)	Glyphosate	2-4-D	Pendimethalin
1:200	0	0	2.8
1:1	0	0	2.6
2: 1	2.3	4	*nt**
3: 1	2.4	4.5	*nt*

*= Not tested, as the bacterium could not be able to resist the herbicide at lower concentrations.
*= Differences were highly significant among the different herbicides at different concentrations.

Production of acids from carbohydrates

Many carbohydrates are utilized by bacteria, with usual production of a considerable extra cellular slime (Gorrity *et al.,* 2001). Tests for ability of the *Agrobacterium* isolate to utilize different carbohydrates, using peptone water sugar, indicated that fructose, lactose, raffinose, sucrose and mannitol could be utilized by this isolate as sources of Carbon, as the colour of

the bromocresol changed from blue to pink (Table 1). Whereas, other carbohydrates such as starch, glucose, galactose and maltose exhibited no change in the color of bromocresol, and they remained to be not fermented by the indigenous isolate.

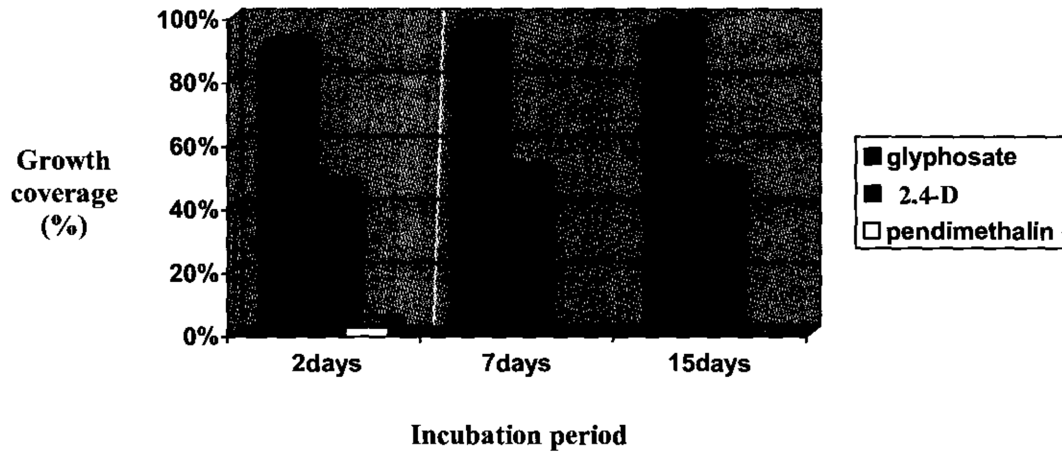

Fig. (1): Effect of incubation period on herbicide resistance.

Fig. (2): Size and shape of tumors produced by Agrobacterium tumefaciens strain SDB0012 on potato discs 21 days after inoculation.

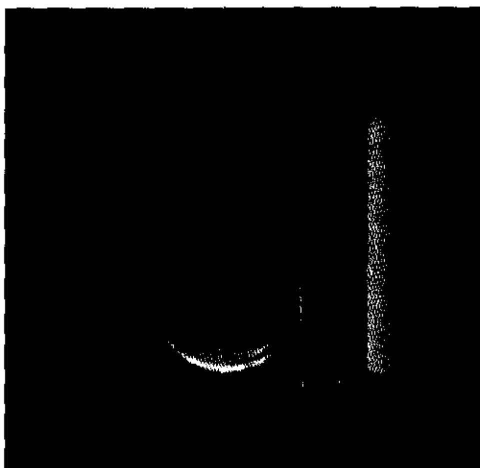

Fig. (3): Response of the indigenous strain to different antibiotics, in the antibiotic disc, 48 hours after inoculation.
Where: 1= penicilin (30mg), 2= chloramphenicol (30mg), 3= ceprofloxacin (30mg), 4= gentamycin (30mg), 5= oflaxacin (30mg), 6= tetracyclin (30mg)

E coli ladder Agro

Fig. (4): Whole plasmid DNA of the local isolate of E. coli, the ladder and Agrobacterium tumefaciens "SDB0012".
It shows two plasmids one plasmid of E. coli, the ladder and two plasmids of A. tumefaciens SDB0012

Response to different herbicides

Only pendimethalin inhibited the bacterial growth at zones of 2.8 cm and 2.6 cm using the dilution rates of 1 herbicide:200 H_2O and 1:1, respectively (Table 2). The bacterium showed resistance to glyphosate and 2,4-D at the two dilution rates. The two herbicides are sprayed in the field at the dilution rate of 0.002 (Personal communication) which is less than the dilution rate of 1:200 used in this experiment with increasing the concentration of glyphosate and 2,4-D, to 1 herbicide :2 H_2O, the bacterium showed inhibition zones of 2.3 and 4 cm, respectively. The inhibition zones increased to 2.4 cm for glyphosate and 4.5 cm for 2,4-D at the dilution rate of 1 herbicide: 3 H_2O . These results indicated that resistance of this strain to glyphosate was better than that of 2,4-D despite resistance to both herbicides was satisfactory and could encourage identification of genes conferring resistance to both herbicides in the chromosomal DNA of the bacterium since genetic resistance of herbicides is considered as an effective mean of weed control. Glyphosate resistance gene (s) was commonly identified in *Agrobacterium* (Wikipedia, 2010). Glyphosate is the active ingredient of roundup. It controls weeds by inhibiting the synthesis of aromatic amino acids necessary for protein formation in susceptible plants. It is strongly adsorbed to soil particles, which prevents it from excessive leaching or from being taken-up from the soil by non target plants (Owen and Zelaya, 2005). Concerning the effect of incubation period on herbicide resistance, the growth coverage of the bacterium on NASA medium was found to be more than 90, 46 and 3/% for glyphosate, 2,4-D and pendimethalin, respectively, after two days of incubation. The growth of *Agrobacterium* was not affected by increasing incubation time to seven and 15 days with glyphosate and 2,4-D, as shown in Figure (1). In contrast, drastic decrease in resistance to pendimethalin was observed as the incubation period increased. These results indicated stability of resistance to glyphosate and 2,4-D.

Type and number of tumors

The addition of bacterial suspension to small discs of potato (1.5 cm in diameter) resulted in growth 4 to 5 big tumors 21 days after inoculation in the three replications (Figure 2). The observed tumor showed shoots like growth on the surface, which indicated that *Agrobacterium* possesses nopaline-type plasmids. This result was in a line with Stephen and Rick (1981) who stated that octopine-type plasmids usually develop unorganized tumors, while nopaline-type plasmids are generally associated with teratoma-like tumors in which small shoots develop on the surface of the tumors.

Plasmid characteristics

Antibiotic resistance is a character controlled gene(s) located in the plasmids of bacterial cell. The bacterial plasmids are known to carry gene(s) of resistance to one or more of known antibiotics such as penicillins, cephalosporins, aminoglycosides (streptomycin, kanamycin, amikacin, gentamicin and tobramycin), tetracycline, polymyxins, rifampicin, fucidin, chloramphenicol, erythromycin, clindamycin and vancomycin (Citz *et al.*, 2005).

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. It is a specific type of drug resistance. Such type of resistance evolves naturally *via* natural selection through random mutation, but it could also be engineered. Results obtained in this study revealed that the indigenous isolate of *Agrobacterium* was completely sensitive to gentamycin (5mm zonat), oflaxacin (3mm zonat) and tetracyclin (3mm zonat) and resistant to penicillin, chloramaphenicol and ceprofloxacin, with no

zonation in growth of *Agrbacterium* around these antibiotics (Figure 3). These results concluded that the plasmid of this strain contained genes conferring resistance against penicillin (30*mg*), chloramphenicol (30*mg*) and ciprofloxacin (30*mg*). Accordingly, the indigenous *Agrobacterium* was described as a multi-resistant or a superbug bacterium since it possesses resistance to more than one antibiotic. An example of multi-resistant bacteria is *Enterococcus faecium* (Jikia *et al.*, 2005). Regarding number of plasmids in the bacterial cell, results indicated that the local isolate of *Agrobacterium* "SDB0012" had two plasmids of more or less similar molecular weight ranging between 2.0- 2.4 kb (Figure 4). Whereas, the indigenous isolate of *E. coli* showed one plasmid of less than 2.0 kb. Presence of two plasmids in the bacterial cell of SDB0012 might explain why this bacterium possesses more than one antibiotic resistance gene.

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النشاط الانزيمي, مقاومة مبيدات الحشائش والخواص البلازميدية للبكتيريا الزراعية *Agrobacterium tumefaciens* السلالة *"SDB 0012"*

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اجريت عدة دراسات بيولوجية و جزيئية على السلالة المحلية من البكتيريا الزراعية *Agrobacterium tumefaciens*" SDB0012 " بالمعهد القومي لتنمية الصادرات البستانية -جامعة الجزيرة- السودان في العام 2005. حيث شملت الدراسة مقدرة البكتيريا للتعبير عن الانزيمات, انتاج الاحماض من المواد الكربوهيدراتية, مقاومة مبيدات الحشائش, الحساسية للمضادات الحيوية, نوع التيومر و خواص البلازميد. اظهرت الدراسة مقدرة هذه السلالة في انتاج انزيمات الاكسيديز oxidase, ويوريز urease و كاتاليز catalase. كما اظهرت مقدرة على استخدام الفركتوز, واللاكتوز, والرافينوز,و السكروز ,و المانتول كمصادر للكربون بخلاف النشا والجلوكوز, والجلاكتوز و المالتوز. اثبتت الدراسة أيضاً مقدرة هذه السلالة على مقاومة مركبي الجلايفوسيت (المادة الفاعلة في مبيد الراوند أب) و2,4-D وحساسيتها لمبيد بنديميثان bendimethan المادة الفاعلة في مبيد الإستومب. كما أظهرت النتوات القائمة على سطح اقراص البطاطس نموات فرعية مما يوضح وجود بلازميد النوبالين في هذه البكتيريا. اظهرت السلالة مقاومة لكل من البنسلين,و الكلورومفنكول و السيبروفلاكسين وحساسية للمضادات الاخري التى تمت دراستها, لذا تم تعريف هذه السلالة بمتعددة المقاومة للمضادات الحيوية او ما يسمي بالسوبر بق Superbug bacteria. كما أوضحت النتائج وجود إثنان من البلازميدات فى خلية هذه البكتيريا متقاربة فى أوزانها الجزيئية (kb 240-200), بينما أظهرت سلالة محلية أخرى من بكتيريا *Escherichia coli* وجود بلازميد واحد ذو وزن جزيئ اقل من بلازميدات البكتيريا الزراعية المستخدمة في هذه الدراسة, مما قد يفسر ظاهرة وجود مقاومة لاكثر من مضاد حيوي في هذه البكتيريا.