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Induction of sporulation and antibacterial activity in the aerial mycelium negative mutants of *Streptomyces nasri*

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ABSTRACT

Induction of sporulation and antibacterial activity in 24 spontaneous (SP), ultraviolet (UV), nitrosoguanidine (NG) and ethidium bromide (ETH) aerial mycelium negative mutants (Bld^{+}) of *Streptomyces nasri* (Hashem & Diab) was investigated. Calcium and phosphate ions could overcome the problem of the development of the aerial mycelium negative mutants and restored sporulation and antibacterial activity. The positive role of calcium was further confirmed with the use of the chelating agent Ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Mutants demonstrated variable restoration of sporulation and antibacterial activity responses toward different concentrations of calcium and phosphate ions. Sporulation induction showed a parallelism to the antibacterial activity exerted by the mutants. On the other hand, some amino acids were found to have a positive effect on sporulation and antibacterial activity. Histidine was the best stimulant for both sporulation and antibacterial activity for SP, and NTG mutants and had the lowest effect on UV and no effect at all on ETH-mutants. Glycine induced sporulation for the UV and ETH-mutants, and phenylalanine and proline in the case of ETH-mutants. Cluster analysis showed a high degree of relatedness between NTG and ETH-mutants in their response to calcium addition. UV and ETH-mutants were highly related when phosphate feeding is the criterion. Internal relatedness among each group also emerged when amino acid addition was evaluated.

KEYWORDS: *Streptomyces nasri*, sporulation, antibacterial activity, aerial mycelium, mutagenesis.

INTRODUCTION

Actinomycetes have been described as the greatest source of antibiotics since Waksman introduced Streptomycetes into his systematic screening program for new antibiotics in the early 1940s. They have provided about two-thirds of the naturally occurring antibiotics discovered, including many of these important in medicine (Okami & Hotta 1988). Gram-positive bacteria within the genus *Streptomyces* are of considerable interest for their morphological development and ability to produce a variety of secondary metabolites (Paquet *et al*. 1992). Streptomycetes are usually cultivated in nutritionally rich media, and antibiotics are sometimes produced in nutritionally limited media that begin to accumulate at idiophase. Since antibiotics can be produced under limited culture conditions, their biosynthesis is subjected to various regulatory mechanisms (such as carbon and nitrogen regulation) that significantly influences antibiotic production (Demain *et al*. 1981). The effect of various metal ions on secondary metabolism has been reported (Weinberg 1970). Ca^{+2} dependent antibiotic production was reported for *S. coelicolor* (Lakey *et al*. 1983), *S. albogriseolus* 444 (Danova *et al*. 1997) and *S. hygroscopicus* 155 (Moncheva *et al*. 2000). Phosphate is another important factor in the synthesis of a wide

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range of antibiotics (Martin 1977) and it has been suggested that phosphorylated intermediates are of prime importance as control elements.

Aerial mycelium formation and sporulation are two important morphological events in the life cycle of actinomycetes. These cytodifferentiation processes have been found to be closely related to the production of secondary metabolites (Redshaw *et al*. 1979; Ginther 1979; Umeyama *et al*. 1999 & Molle *et al*. 2000). Several differences in the ribosomal protein pattern of substrate and aerial mycelium of *S. antibioticus* were recorded by De-Los-Reyes Gavilan *et al*. (1991). These differences suggest that during cell differentiation in *Streptomyces*, important changes occur at the ribosomal level, particularly in the transition from substrate to aerial mycelium.

The nature of the most frequently occurring mutations depends to a large extent on the procedure used to induce it. Mutagenesis itself is considered to be the experimental increase of the spontaneous mutation rate (Lengeler *et al.* 1999). Although spontaneous mutation occurs in the fermenter, other mutagenesis routes can not be ignored as a result of the biochemical stress. The development of the non-sporulating areial mycelium during the fermentation of *Streptomyces* strains poses problems and causes an economic loss in the industrial production of antibiotics by this genus. The major objective of this work is to monitor and manipulate the emergence of these mutants in a trial for the restoration of the sporulation potential and the antibacterial activity through the addition of Ca^{2+} either in the presence or absence of EGTA, as well as phosphate and amino acids to the culture medium.

MATERIALS AND METHODS

Micro-organism and maintenance: *Streptomyces nasri* (Hashem and Diab) was isolated from the desert of Kuwait by Hashem & Diab (1973). This strain was found to produces an aromatic antibiotic active against Gram-positive bacteria. For the maintenance of this strain, nutrient agar medium was used and the slants were kept at 5°C until further use.

Mutagenesis: Morphologically stable (after 6 transfers on oat meal agar) Bld^+ mutants of the S. nasri parent strain were obtained spontaneously where the mutants were isolated from the sectors formed at the periphery of old colonies (14 days). Ultraviolet (UV) mutagenesis was carried out according to Zhang et al. (1993). Nitrosoguanidine (NTG) mutagenesis was performed according to Holmalahti *et al.* (1993). Ethidium bromide (ETH) mutagenesis was applied as described by Dary et al. (1992). In all mutagenesis techniques, photoreactivation was avoided by keeping the isolated colonies overnight in the dark by wrapping the plates in aluminum foil (Held & Kutzner 1991) and a survival rate of not more than 1% was considered. All mutants failed to sporulate or to produce an anti-S. aureus effect when grown on oatmeal agar.

Culture conditions and sporulation: Oatmeal-liquid medium as described by Küster (1959) was used for antibiotic production to which agar (Difco) was added (18g/l) to prepare plates for the colony and spore development; its pH was adjusted to 7.3 before sterilization at 1.5 atm., 121°C for 15 min. The calcium and phosphate contents of oat meal agar medium was determined to be 0.02 and 1.0 mM/l, respectively by atomic absorption analysis (Cornning Clinical Flame Photometer 410C). This was taken into consideration when calcium and phosphate salts were added. Calcium ($CaCO_3$) and phosphate (KH_2PO_4) salts were separately added to the liquid and solid medium, so as to make a concentration range of 0.05-2.0mM/L for calcium and 1.0-22 for phosphate. For the amino acid feeding experiment, 11 amino acids representing different families were used, each individually added to the oatmeal medium (glutamic acid, tyrosine, leucine,

proline, phenylalanine, glycine, asparagine, lysine, alanine, tryptophan, histidine) in the presence of the optimum calcium and phosphate concentration for each mutant. The amount of each amino acid was equivalent to the nitrogen content of 2.0g $NaNO_3$. Ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) was only added to oatmeal media containing calcium carbonate. The pH of EGTA solution was originally adjusted to 7.3. Oat meal agar medium was poured in Petri-dishes (9-cm diameter each) each received 20 ml medium and inoculated with 0.5 ml of *Streptomyces nasri* spores ($4x10^6$) and incubated at 30°C for 14 days. Using a cork borer (6 mm inner diameter), 4 plugs were removed from each sporulating colony (14 day old) 0.5cm apart from the centre of the colony. The plugs were then transferred into test tubes containing 1:1:4 (70% ethanol: glycerol: water) mixture and shaken gently to prepare spore suspension to make a 5ml final volume; 2ml of this suspension were measured at 580nm using Spekol 11 colorimeter. Readings represent the turbidity (%) relative to the turbidity of the spore suspension of the parent strain which was considered to be 100%. Experiments were repeated three times and the mean value taken.

Bioassay*:* To test for the antibacterial activity, Staphylococcus aureus ATCC 25923 was used as a test organism. Mueller-Hinton agar medium was used as an assay medium. The agar medium at 45°C was mixed with 0.1 ml bacterial suspension containing approximately 10^5 cfu/ml. The mixture was poured into 9 cm Petri dish and allowed to solidify. Sterile paper discs (6mm) were placed on the dried surface of the medium. Each disc received 20 µl of the culture filtrate. Petri dishes were incubated at 37°C for 18 hours. The inhibition zone, if any, was measured in mm diameter (Amade *et al.* 1994).

Cluster analysis: Statistica version 5 was used for the cluster analyses using complete linkage and Euclidean distances.

RESULTS

Restoring sporulation of the aerial mycelium negative mutants of *S. nasri* after their treatment with different Ca^{2+} concentrations occurred at 0.1-0.6mM/L for SP mutants group (Fig. 1a), 0.8-1.4 mM/L for UV mutants group (Fig. 1b), 0.1-1.4 mM/L for NG mutants group (Fig. 1c) and 1.4-2.0 mM/L for ETH-mutants group (Fig. 1d). The anti-*S. aureus* activity (Fig.2a-d) was more or less similar to the sporulation behaviour, with only one exception, is that ETH-mutants No. 1 and 2 produced their antibacterial activity at a lower Calcium concentration (0.2 & 0.4 mM/L) compared to the other mutants of the same group (Fig.2d). In control medium when calcium was omitted, sporulation failed to occur and no antibacterial activity was detected for any of the four mutant categories.

To confirm the role of calcium, the inhibition percentage against control after the addition of EGTA (5 µmole/ml) was calculated. Since the maximum sporulation occurred at calcium concentrations of 0.2, 0.4 mM/L for spontaneous mutants, 1.0 - 1.2 mM/L for UV and Nitrosoguanidine and 1.6, 1.8 mM/L for Ethidium bromide mutants, the addition of EGTA was only investigated at these particular concentrations. SP mutants showed 100% sporulation inhibition and all the mutants were aerial-mycelium negative. Other groups of mutants showed variable responses. Sporulation of UV-mutants was reduced by 55-71%, whereas nitrosoguanidine and ethidium bromide mutants sporulation reduced by 72-86% and 20-36%, respectively. Since the sporulation failed to occur, the activity was not detected against *S.aureus* for spontaneous mutants and inhibited by 32-61% for UV mutants compared to the control (with no chelating agent added). Inhibition of activity for nitrosoguanidine-derived mutants was 78-

94% and for ethidium bromide-obtained mutants was 22-66%. The results shown in Figure 3 demonstrate the effect of phosphate addition on sporulation response.

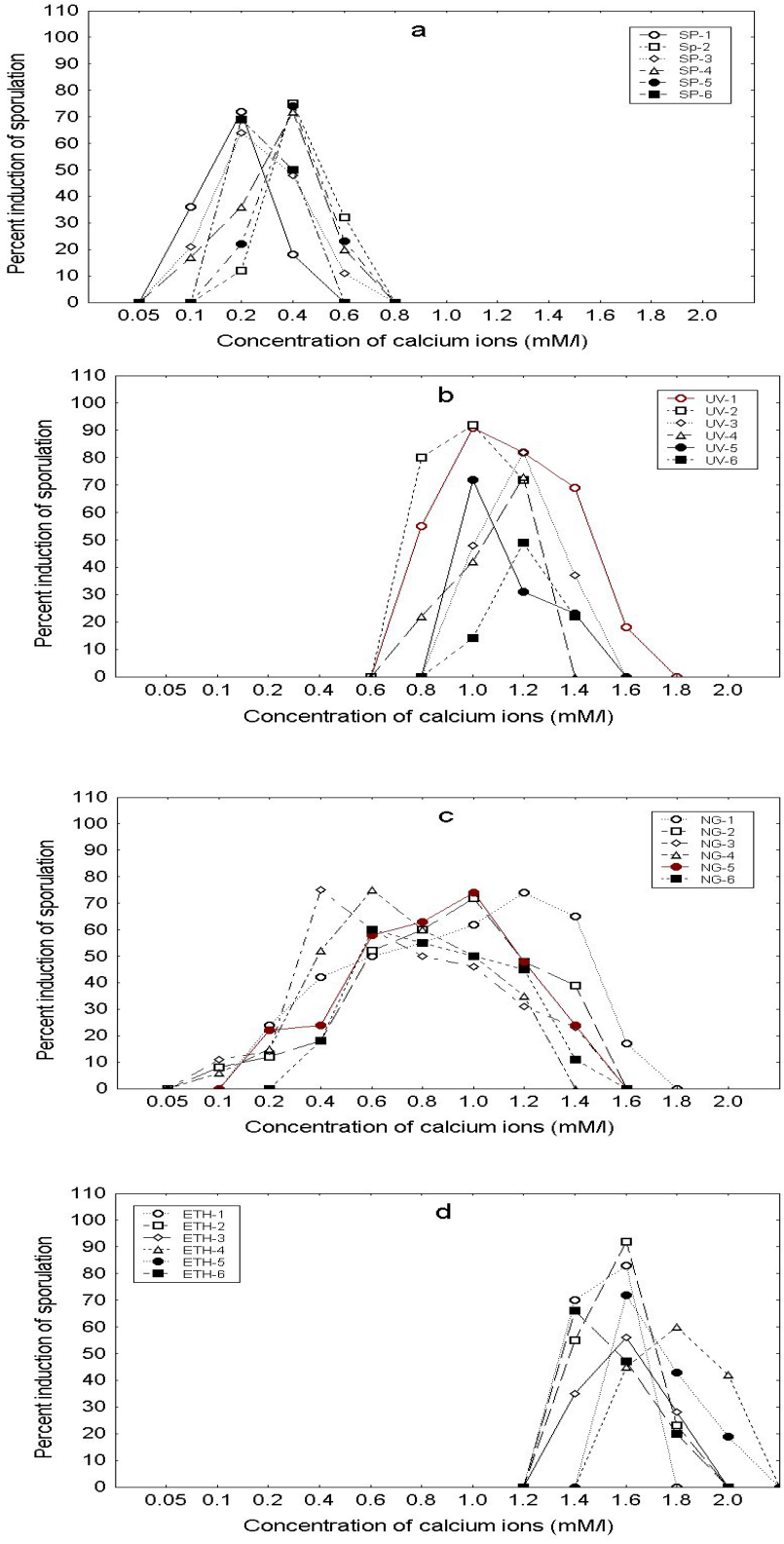

Figure1: Effect of different calcium ion concentrations on sporulation a) spontaneous, b) ultraviolet, c) nitrosoguanidine and d) ethidium bromide mutants of *S. nasri*

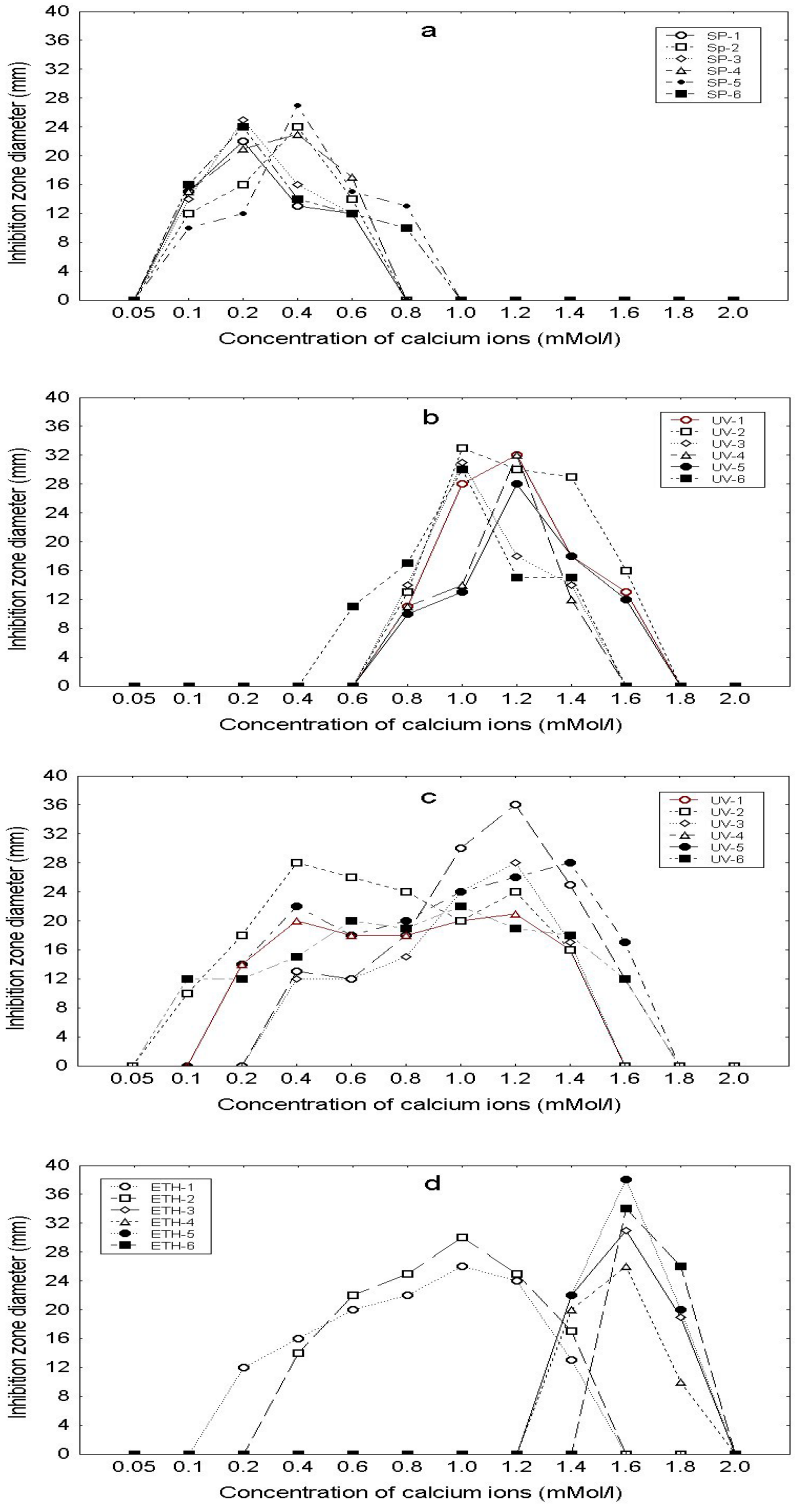

Figure 2 Effect of different calcium ion concentrations on anti-*S. aureus* activity of a) spontaneous, b) ultraviolet, c) nitrosoguanidine and d) ethidium bromide mutants of *S. nasri.*

Spontaneous mutants (Fig.3a) were the most sensitive to phosphate addition since they sporulated at a wide phosphate range of 2.0-18 mM/L compared to 2.0-12 for the UV category, 14-22 for the NG group and 4-12 mM/L for the ETH-mutants.

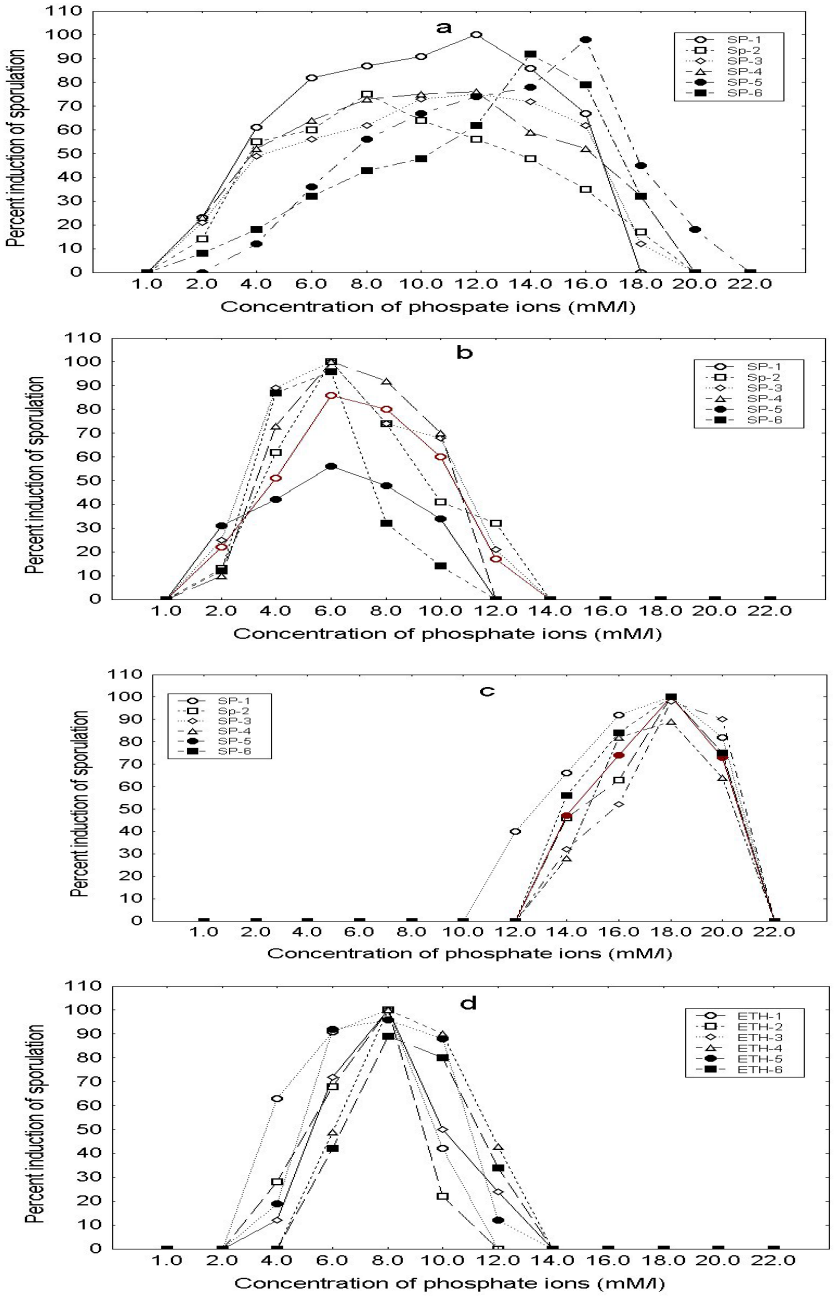

Figure 3 Effect of different phosphate ion concentrations on sporulation of a) spontaneous, b) ultraviolet, c) nitrosoguanidine and d) ethidium bromide mutants of *S. nasri.*

It was interesting to observe that the maximum sporulation induction for SP mutants (Fig. 3a) occurred at high phosphate levels (14-16 mM/L), whereas antibacterial activity maxima (Fig. 4a) for SP3, SP4 & SP6 was around 4 mM/L, SP2 and SP5 at 6mM/L, SP1 at 8 mM/L. The antibacterial response of the other groups was quite similar to the sporulation pattern (Figs. 4b-d).

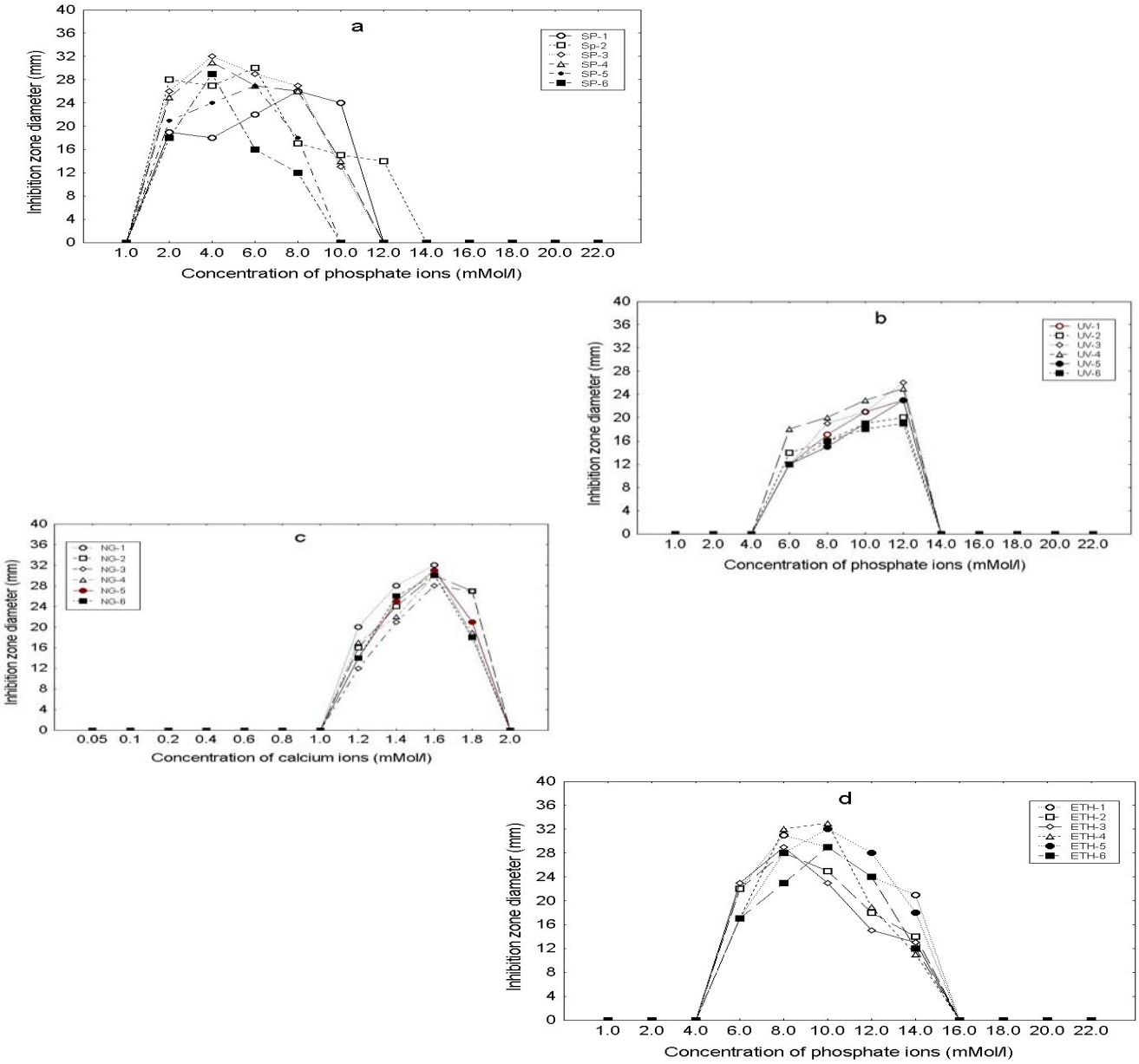

Figure 4. Effect of different phosphate ion concentrations on the anti-*S. aureus* activity of a) spontaneous, b) ultraviolet, c) nitrosoguanidine and d) ethidium bromide mutants of *S. nasri*.

The degree of relatedness (expressed as a linkage distance) between *S. nasri* mutants categories was higher for antibacterial activity (68 for calcium and 80 for phosphate) than sporulation (170 for calcium and 252 for phosphate). The cluster analysis for sporulation induction by calcium resulted in a dendrogram with two symmetric major clusters. The first major cluster included two sub-clusters for SP and ETH-mutants (Fig. 5). The second contained UV and NG groups. Similar clusters were obtained for antibacterial activity except for ETH-mutants where the behaviour showed more relatedness to NG. The dendrograms of phenotypic data for phosphate showed that SP, UV and ETH-mutants were included in one major related cluster, whereas NG remained in a separate entity with the lowest degree of relatedness to the other groups. Overlaps exist between UV and ETH-mutants. It is worthy to record the consistent behviour of NG mutants, where they

tend to occupy one separate relatively unrelated cluster when sporulation and antibacterial activity with phosphate was the criterion.

The data in Table 1 showed that histidine and proline could induce sporulation in the four mutant groups. The rest of the amino acids differentially induced spore formation, but alanine and aspartic acid recorded the lowest sporulation percentage. 100% sporulation record was achieved with histidine in SP mutants, glycine in UV mutants, histidine in NG mutants, proline, glycine, phenylalanine and histidine in ETH-mutants. Generally, the data demonstrated that anti-*S.aureus* activity was more or less directly proportional to sporulation and that there was a threshold beyond which no activity detected. Glycine was the only amino acid that contradicted this trend. Moreover, on the one hand, alanine failed to stimulate anti-*S. aureus* activity in any group of mutants, but on the other hand, histidine could restore the activity in all mutant groups. Amino-acid addition produced a different relatedness profile in comparison to that obtained from calcium, but was closely similar to the phosphate dendrograms. The dendrogram (Fig. 6) showed a close relatedness between UV and ETH-mutants and to a lesser extent to SP mutants. These three groups are included in one cluster highly unrelated to NG mutants.

DISCUSSION

In actinomycetes, a close relationship between secondary metabolism and cell differentiation has been established and exogenous metabolites can positively regulate the production of antibiotics. Extracellular stimulation during differentiation in *Streptomyces* involves metabolic compounds as endogenously produced diffusible factors, and the synthesis of morphogenic proteins that direct the morphogenesis of aerial mycelium (Champness & Chater 1994). Among these are A-factor (2-isocapryloyl-3R-hydroxy-methyl γ-butyrolactone) and SaPB (small aerial mycelium proteins), both associated with aerial mycelia and spores of *S.coelicolor* A3(2). Calcium and GTP have been proposed to play an important role in regulating the bacterial ll cycle (Lengeler *et al.* 1999). Ochi (1987) emphasized the importance of the level of GTP and its hyper-phosphorylated derivatives in controlling the synthesis of A-factor and thereby streptomycin production by *Streptomyces griseus* (Hara & Beppu 1982). Hobbs *et al*. (1990) found that actinorhodin production was completely inhibited by 24mM-phosphate, whereas undecylprodigiosin was still formed at this concentration. They contributed the differential sensitivity of undecylprodigiosin and actinorhodin to phosphate to a direct interaction with the biosynthetic enzymes. On the other hand, Hatada *et al*. (1994) demonstrated that the cultivation of *Streptomyces griseus* strain 2247 under stress conditions for growth generated pleiotropic mutants at high frequency. These mutants had lost simultaneously streptomycin productivity, streptomycin resistance, spore forming ability and pigment productivity, although the genes for streptomycin biosynthesis and A-factor production were proficient.

In the culture medium used for the growth of actinomycetes, $CaCO_3$ is mostly one of the ingredients and its role was originally to maintain the neutral pH conditions. Natsume *et al.* (1989) described another role in the induction or stimulation of cytodifferentiation such as aerial mycelium formation. The results obtained in this paper showed differences in the calcium concentration requirement for aerial mycelium formation according to the mutant's origin. The calcium requirement for SP, UV and NTG mutants coincides with the actinomycetes extracellular Ca^{2+} requirements for the aerial mycelium formation that ranged from 0.1-1.5 mM. The effect of Ca^{2+} on differentiation of *S. hygroscopicus* 155-0 was scrutinized by Moncheva *et al*. (2000) who found that addition of calcium to the culture medium induced the aerial mycelium formation in an inactive variant and accelerated its formation in the parent strain. They reported the inhibitory

effect of verapamil, nifedipin, chlorpromazine and dilthiazme addition on calcium uptake. In contrast, calcium addition to *S. albogriseolus* 444 culture had no significant effect on the negericin production, although the biomass accumulation increased (Danova *et al*. 1997).

Collectively, the results obtained here confirm the necessity of calcium and phosphate for induction of sporulation and antibacterial activity. This may be due to a role for these ions in the biosynthesis of the A-factor or in the activation of enzymes involved in the synthesis of SaPB to restore both sporulation and antibiotic production by *S. nasri* mutants. These two putative regulatory systems have been implicated in the secondary metabolism of *Streptomyces* spp., as described by Shizuka *et al*. (1992). Amino acid addition could have a positive role in cytodifferentiation in this strain, since the addition of glycine, leucine and aspartic acid had a clear enhancement for both sporulation and antibacterial activity. This may relate to the formation of SaPB, which contains these amino acids in its structure. The phenotypic relatedness could be also used to eliminate duplicate actinomycete strains in the future by a microbial screening programme. Further investigations should definitively answer the question of how secondary metabolism and aerial mycelium development are regulated in this strain. The effects of A-factor and SaPB on the cytodifferentiation are being currently investigated.

Table 1. Percent induction of soporulation and anti-Staphylococcus activity of the bld+ mutants of *Streptomyces nasri* after treatment with different amino acids in presence of the optimum Ca^{2+} and PO_4 concentration for each mutant.

Mutant Number	Amino acid																			
	Glu		Pro		Asp		Lys		Try		Pha		Tyr		Gly		Ala			
	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ	Spo	IZ		
SP-1	74	*12*	89	*30*	-	-	26	*23*	83	*28*	-	-	70	*18*	32	*28*	13	-		
SP-2	82	*23*	94	*32*	-	-	29	*29*	96	*32*	-	-	59	*10*	34	*30*	10	-		
SP-3	69	*19*	78	*26*	-	-	32	*32*	90	*30*	-	-	62	*13*	28	*32*	22	-		
SP-4	78	*20*	82	*28*	-	-	24	*24*	92	*31*	-	-	73	*15*	31	*30*	17	-		
SP-5	81	*22*	87	*27*	-	-	30	*30*	98	*33*	-	-	54	*10*	41	*28*	12	-		
SP-6	77	*21*	90	*31*	-	-	27	*29*	86	*21*	-	-	48	*14*	35	*33*	23	-		
UV-1	56	*16*	10	-	18	*18*	-	-	-	-	80	-	90	*30*	100	*33*	12	-		
UV-2	64	*14*	22	-	28	*28*	-	-	-	-	72	-	86	*24*	100	*32*	10	-		
UV-3	71	*18*	15	-	20	*20*	-	-	-	-	68	-	83	*23*	100	*32*	15	-		
UV-4	54	*17*	18	-	31	*22*	-	-	-	-	62	-	76	*19*	100	*33*	18	-		
UV-5	68	*13*	21	-	24	*24*	-	-	-	-	81	-	84	*28*	100	*34*	22	-		
UV-6	47	*18*	24	-	22	*22*	-	-	-	-	90	-	94	*31*	100	*35*	13	-		
NG-1	-	-	88	*32*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NG-2	-	-	90	*28*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NG-3	-	-	83	*33*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NG-4	-	-	89	*30*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NG-5	-	-	77	*32*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NG-6	-	-	85	*29*	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
ETH-1	17	-	100	*32*	-	-	-	-	23	*21*	100	*21*	55	-	100	*32*	-	-		
ETH-2	12	-	100	*29*	-	-	-	-	38	*23*	100	*32*	53	-	100	*27*	-	-		
ETH-3	10	-	100	*25*	-	-	-	-	32	*22*	100	*28*	61	-	100	*29*	-	-		
ETH-4	18	-	100	*31*	-	-	-	-	41	*21*	100	*32*	65	-	100	*28*	-	-		
ETH-5	20	-	100	*28*	-	-	-	-	36	*24*	100	*28*	61	-	100	*31*	-	-		
ETH-6	12	-	100	*30*	-	-	-	-	12	*16*	100	*30*	58	-	100	*32*	-	-		

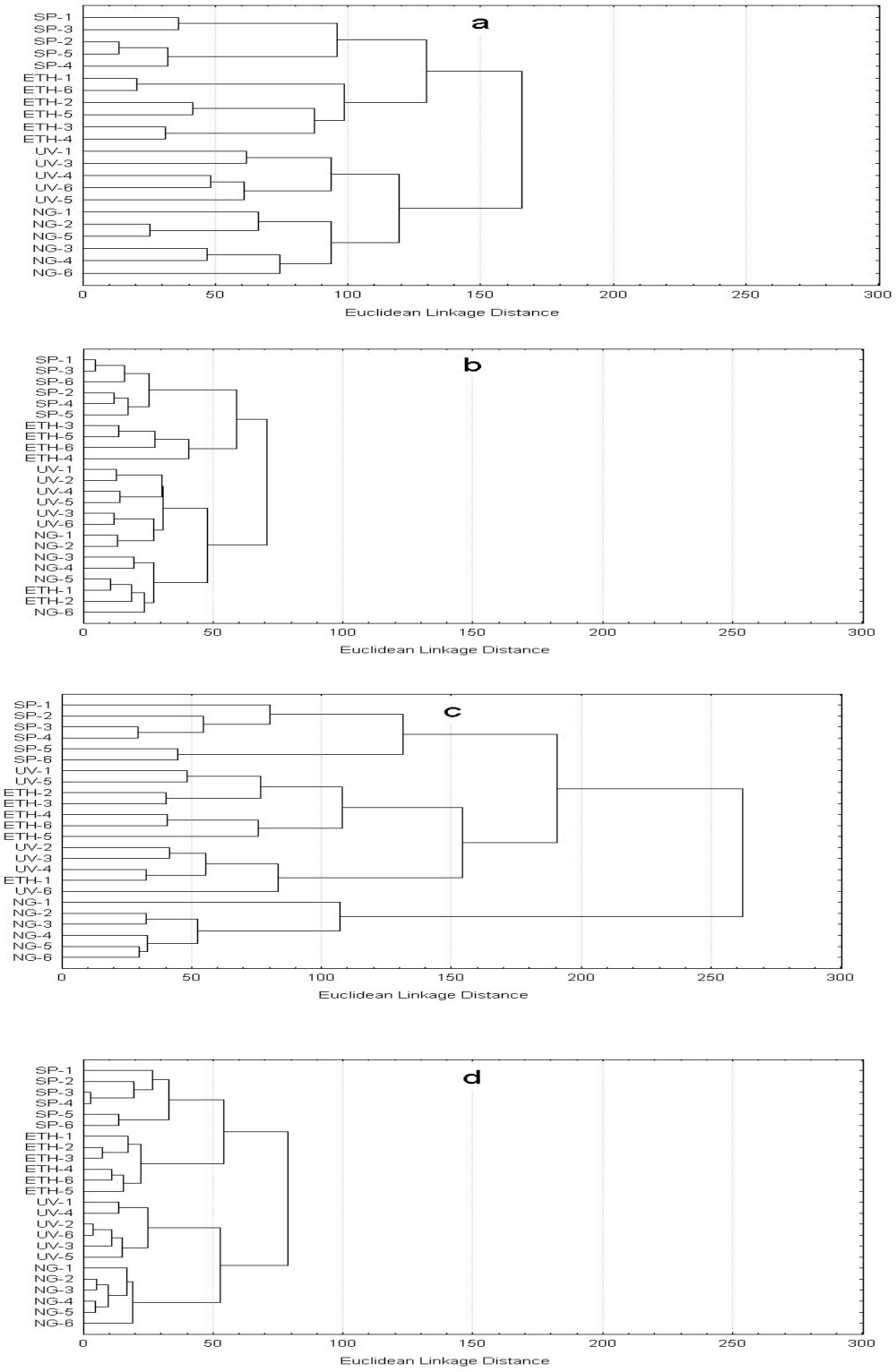

Figure 5 Dendrogram showing the grouping profile of *S. nasri* sporulation (a & c) and antibacterial activity (b & d) responses after calcium and phosphate addition, respectively.

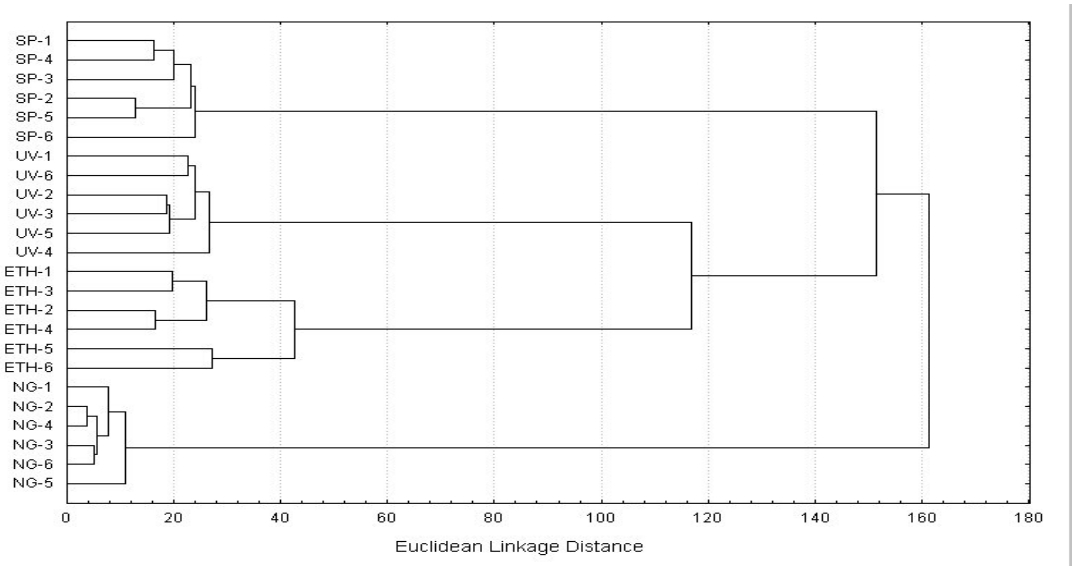

Fig. 6. Cluster analysis output (dendrogram) for the sporulation percentages and anti-*S. aureus* activity of the 24 Bld+ *Streptomyces nasri* mutants supplemented with different amino acids.

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الملخص العربى

دفع التجرثم والنشاط ضد البكتيري في طفرات *ستربتوميسيس نصري* سالبة الغزل الهوائي

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استهدفت الدراسة حل مشكلة إخفاق عملية إنتاج المضادات الحيوية في عمليات التخمرات الصناعية وذلك نتيجة ظهور طفرات سالبة الغزل الهوائي مما يسبب خسائر اقتصادية كبيرة. استخدم في هذه الدراسة طفرات مختلفة مستحدثة من البكتيرة *ستربتوميسيس نصـري* (المنتجـة لمـواد ضـد-*ستافيلوكوكس أورياس*) باستخدام مادتي نيتروزوجوانيدين وبروميد الإيثيديم بالإضافة إلي الأشعة فوق البنفسجية. وأضيف إلـي هـذه المجموعـة عزلات من طفرات تلقائية. وقد استخدم في عملية الدفع تركيزات محددة من أيونات الكالسيوم والفوسفات بالإضافة إلى بعض الأحماض الأمينيـة.

وتم التأكيد على الدور الإيجابي لأيونات الكالسيوم باستخدام مادة إى.جي.تى.إيه. ومما هو جدير بالذكر أن استجابة الطفرات للتجرثم كانت موازيـة لعملية دفع النشاط ضد البكتيري في حالات استخدام أيونات الكالسيوم والفوسفات. أما تجريب الأحماض الأمينية فقد أوضح فاعلية حمض هيستيدين مع الطفرات التلقائية وطفرات نيتروزوجوانيدين بينما كان حمض جليسين هو الفعال مع طفرات الأشعة فوق البنفسـجية وبروميـد الإيثيـديم. وأن أحماض فينيل الآنين وبرولين كان تأثيرهما ملحوظا فقط مع طفرات بروميد الإيثيديم. هذا وقد عضدت النتائج إحصائيا باستخدام طريقة المجموعات العنقودي.