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Conditions for the production of jadomycin B by *Streptomyces venezuelae* ISP5230: effects of heat shock, ethanol treatment and phage infection

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SUMMARY

The novel benzoxazolophenanthridine antibiotic, jadomycin B, is produced by *Streptomyces venezuelae* ISP5230 following a 42 °C heat shock or exposure to ethanol. To further characterize these unusual culture conditions, studies were carried out using different media, varying nutrient content and concentrations, initial pH, and time of application of heat or ethanol stress. Highest titers of jadomycin B accumulated 48 h after *S. venezuelae* ISP5230 was inoculated into a D-galactose-L-isoleucine production medium (pH 7.5) which was supplemented with ethanol (6%, v/v) between 6 and 13 h. Cultures supplemented with ethanol later than 17 h post inoculation into the production medium produced little or no jadomycin B. Among other heat-shock inducing treatments examined, infection with phage SV1 was associated with increased jadomycin B production. Although jadomycin B titers showed little change with variations in the concentration of phosphate in the production medium, the nature of the nitrogen source was found to be important. Different colored pigments, presumed to be jadomycin B analogs, were formed when other amino acids replaced L-isoleucine in the medium as the sole nitrogen source. Increased jadomycin B titers accompanied increased L-isoleucine and D-galactose concentrations in the production medium.

INTRODUCTION

In a variety of *Streptomyces*, growth suppression due to exhaustion of carbon, nitrogen, or phosphate from the culture medium often allows for expression of the genetic information for antibiotic biosynthesis [5]. In some cases, this expression has been linked mechanistically to regulons, such as the Pho system, responsible for detecting and adapting the cell to available sources of nutrients in the environment [14]. Some common stress proteins are reportedly induced in response to either nutrient limitation or cell damage caused by physical agents, such as high temperature, indicating a degree of overlap in genetic systems responding to multiple forms of stress [13]. Despite this, the association between antibiotic biosynthesis and non-nutritional stresses such as heat shock has not been investigated. The discovery of multiple *gro*EL-like genes in a variety of streptomycetes, however, has prompted the suggestion that heat-shock-induced chaperones might play a role in the assembly of multienzyme complexes for antibiotic biosynthesis [9]. Recent studies have also indicated that DnaK and other highly conserved proteins associated with heat stress may be involved in the morphological development of *Streptomyces* [3].

We have recently found that *Streptomyces venezuelae* ISP5230, a chloramphenicol producer, synthesizes a group of novel benzoxazolophenanthridine antibiotics, the jadomycins, during continuous growth at elevated temperature [2]. The main component in the mixture of jadomycin derivatives produced during growth of *S. venezuelae* ISP5230 at 37–42 °C on a defined D-galctose-L-isoleucine medium, is a reddish glycosylated product called jadomycin B (Fig. 1). The association of jadomycin B production with high temperature and the virtual absence of the compound from cultures growing at 27 °C suggested that its synthesis might be linked to the heat-shock response, a universal cellular stress response characterized by the production of a specific set of proteins, the heat-shock proteins.

In accordance with this, we observed that although some jadomycin B accumulates during growth of *S. venezuelae* ISP5230 at 37–42 °C, higher concentrations are detected after cultures had been shifted back to 27 °C following a 1 h 42 °C heat shock [6]. Under these fermentation conditions, jadomycin B titers increase rapidly during a 12-h period following heat-shock treatment and then slowly decline

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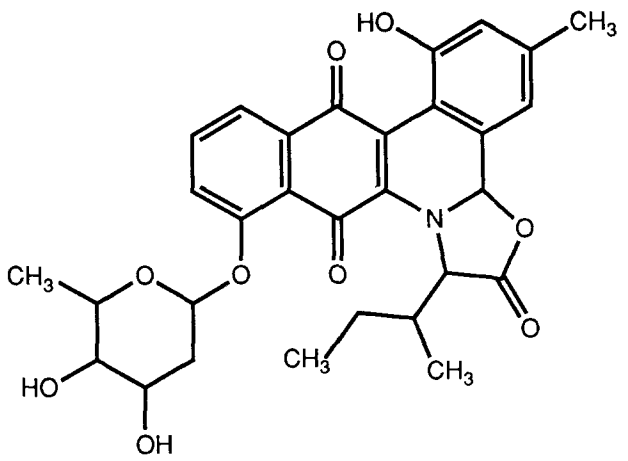

Fig. 1. Chemical structure of jadomycin B (S.W. Ayer et al., unpublished results).

during the following 60 h. Even higher titers accumulate in cultures exposed to ethanol, a known inducer of the heat-shock response in other cell systems [1,12,15]. Under the latter conditions, jadomycin B concentrations increase sharply during the first 24 h after ethanol addition, peak by 48 h, and then slowly decline [6]. Jadomycin B, which inhibits the growth of Gram-positive bacteria and yeast, appears to be the first example of a heat-shock-inducible antibiotic.

In this paper we present data which further characterize the unusual culture conditions promoting the biosynthesis of jadomycin B, with the goal of optimizing its production and gaining insights into its regulation.

MATERIALS AND METHODS

Culture and growth conditions

Streptomyces venezuelae ISP5230 (ATCC 10712) [18] substrain HP-1 (a single colony isolate of ISP5230) used in this study was preserved as spore suspensions at −20 °C in 20% (v/v) glycerol. Vegetative inocula were prepared by incubating 50 μl of spore stock in 100 ml of MYM medium [maltose (0.4%, w/v), yeast extract (0.4%, w/v), malt extract (1%, w/v)] [18] for 20–24 h with shaking (250 r.p.m.) at 27 °C. Unwashed mycelium (5 ml) from these cultures was then added to 50 ml of D-galactose-L-isoleucine medium (pH 7.5), which (unless otherwise stated) contained: D-galactose, 0.1 M (2%, w/v); L-isoleucine, 30 mM; 10 mM-phosphate (potassium dihydrogen phosphate and dipotassium hydrogen phosphate as a 7:3 (w/w) mixture); magnesium sulfate heptahydrate, 0.81 mM; calcium chloride dihydrate, 0.6 mM; sodium chloride, 1.5 mM; ferrous sulfate heptahydrate, 0.032 mM; and a trace mineral solution (4.5 ml L^{-1}) described by Chatterjee and Vining [4].

Following growth for 6–9 h (unless otherwise stated) at 27 °C with shaking (250 r.p.m.) in the D-galactose-L-isoleucine medium, one of the following treatments was applied: i) heat-shock cultures were placed at 42 °C for 1 h with shaking (250 r.p.m.) and then shifted back to 27 °C and grown for an additional 12 h; ii) ethanol treatment — ethanol was added to a final concentration of 6% (v/v) and the cultures were grown for an additional 3–4 days at 27 °C. Both vegetative inocula and jadomycin B production cultures were grown in 250-ml Erlenmeyer flasks.

Infection of cultures with phage SV1

Spore suspensions (80 μl) were added to 50 ml of D-galactose-L-isoleucine medium containing 5 ml of MYM medium. After 2 h growth at 27 °C, a 20-μl suspension of (10^8 pfu ml^{-1}) phage SV1 [18] was added and the culture was incubated for an additional 48 h. Phage SV1 infection of *S. venezuelae* ISP5230 on solid agar was performed according to the method of Stuttard [18], except that a soft nutrient agar overlay was applied to solid D-galactose-L-isoleucine medium containing agar (15 g L^{-1}) and yeast extract (2 g L^{-1}).

Culture broth analyses

Values from 3–9 replicated cultures were averaged. Biomass was measured as the dry weight of cell material collected from samples by filtration. Chloramphenicol was measured as described by Shapiro and Vining [17]. Galactose concentration was determined by the method of Folin and Wu [8]. Phosphate concentration was determined by the method of Herbert et al. [10].

To prepare samples for the jadomycin B assay, 3-ml portions were removed from cultures at intervals and filtered (Whatman No. 1 paper, Whatman International, Maidstone, UK) to remove mycelium. They were then refiltered (Millex-GV, 0.22 μm, Millipore Corp., Bedford, MA, USA) and 1-ml portions were evaporated in vacuo (Savant Speed Vac Concentrator, Savant Instruments, Farmingdale, NY, USA). The residues were resuspended in 200 μl of methanol–water (1:1, v/v), sonicated (three 30-s bursts in a Branson 3200 sonic water bath, Branson Cleaning Equipment, Shelton, CT, USA), and filtered again (Millipore Ultrafree-MC 0.45-μm filtration units). The samples were then assayed using high performance liquid chromatography (Hewlett Packard 1090 HPLC system, Hewlett Packard (Canada), Mississauga, Ont., Canada). Samples (20 μl) were applied to a Vydac (Hesperia, CA, USA) ODS column (250 mm × 2.1 mm i.d.) and jadomycin B was eluted at a flow rate of 0.2 ml min^{-1} with a gradient of acetonitrile/water (50:50 v/v, each containing 0.1% v/v trifluoroacetic acid) to acetonitrile (0.1% trifluoroacetic acid, v/v) over 20 min. Detection was at 313 nm. Jadomycin B eluted at 11.5 min. Jadomycin B concentation in samples was determined by comparison of peak areas to those obtained using standards of known concentrations prepared from purified jadomycin B. As an alternative method of assay, culture samples (5 ml) were collected, mycelium removed by filtration through Whatman No. 5 paper, and Millex-GV 0.22-μm units, and the filtrates passed through 1-ml solid phase extraction cartridges (ODS, Chromatographic Specialties Inc., Brockville, Ont., Canada). After washing with 1 ml water, jadomycin B was eluted with 2.5 ml methanol and the absorbance of the eluate was read at 526 nm using a Hitachi Perkin-Elmer 139

Spectrophotometer, Hitachi, Tokyo, Japan. Jadomycin B concentration was again determined by comparison with standards of known concentrations prepared from purified jadomycin B samples. Determinations of jadomycin B concentrations by the above two methods were in good agreement.

RESULTS

Effects of the time of ethanol or heat treatment on jadomycin B production

Previous observations had suggested that the timing of the heat or ethanol treatment following inoculation into jadomycin production medium may have an effect on jadomycin B titers. The effect of ethanol additions at different times post inoculation was therefore investigated. Ethanol was added to *S. venezuelae* cultures at 6, 9, 13, 17, 24, 48, 72, and 96 h after inoculation. Cultures were incubated at 27°C for a further 96 h and sampled daily. Maximum values were obtained by 48 h post ethanol addition in all cultures (Fig. 2). Cultures that were ethanol treated at 6–13 h produced the highest titers of jadomycin B. Cultures treated 17–96 h after inoculation failed to produce significant amounts of jadomycin B despite the fact that they had accumulated a higher biomass by the time of ethanol addition. Ethanol treatment resulted in the cessation of culture growth within 48 h, regardless of the treatment time, and the amount of biomass present (data not shown).

Similarly, the effect of heat shock at different times post inoculation was investigated by measuring jadomycin B titers 12 h after treatment. This had previously been shown to be the time when jadomycin B titers peaked following heat shock [6]. Figure 3 shows jadomycin B titers obtained in *S. venezuelae* cultures heat shocked at 3, 6, 9, 12, and 15 h after inoculation into D-galactose-L-isoleucine medium. Optimum jadomycin B titers were obtained with the cultures treated at 9 h.

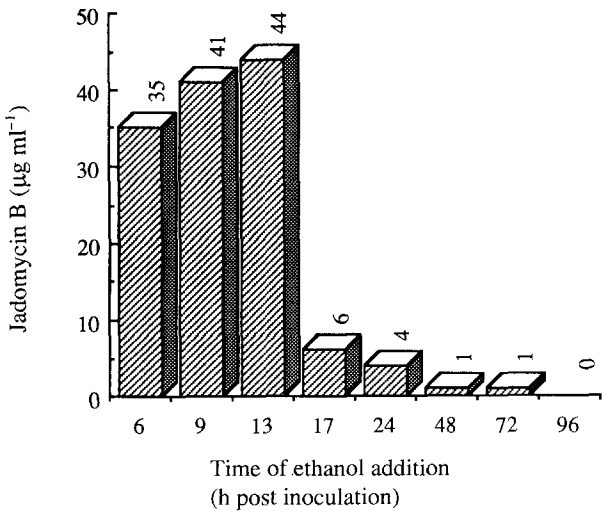

Fig. 2. Jadomycin B concentrations in *Streptomyces venezuelae* cultures supplemented with ethanol (6%, v/v); 6, 9, 13, 17, 24, 48, 72, and 96 h after inoculation into D-galactose-L-isoleucine medium. Jadomycin B titers shown are samples taken 48 h post ethanol addition and are the maximum values obtained over a 4-day period.

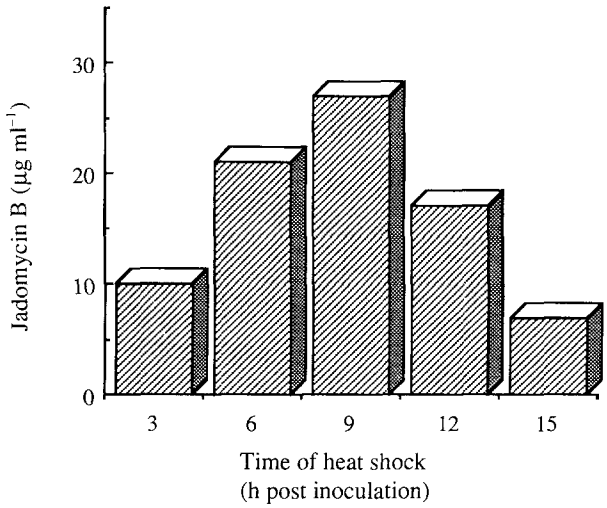

Fig. 3. Jadomycin B titers obtained in *Streptomyces venezuelae* cultures heat shocked for 1 h at 42 °C; 3, 6, 9, 12 and 15 h after inoculation into D-galactose-L-isoleucine medium. Values shown are those obtained at 12 h post heat shock.

Effect of pH and phosphate concentration on jadomycin B production

Since ethanol treatment, rather than heat shock, generally supported the best yields of jadomycin B, the effects on production of varying media constituents were examined in ethanol-supplemented cultures. In order to determine the effect of pH on jadomycin B production, D-galactose (2%, w/v), phosphate (10 mM) and L-isoleucine (30 mM) medium at pH 6, 6.5, 7, 7.5, and 8 was inoculated with *S. venezuelae*. Ethanol (6%, v/v) was added 9 h later and jadomycin B production was monitored every 24 h over a 96-h period. Maximum titers were obtained in all cultures by 48 h with the highest in those at a starting pH of 7.5 or 8. Negligible amounts (less than 4 μg ml^{-1}) were produced in the cultures initially at pH 6 or 6.5. A drop of approximately 1 pH unit was detected in all cultures by the end of the incubation period.

S. venezuelae cultures supplemented with D-galactose (2%, w/v), 30 mM-L-isoleucine, ethanol (6%, v/v) at 6 h post inoculation, and varying concentrations of phosphate (2.5, 5, 10, 20, 40 mM) were adjusted to pH 7.5 and grown for 72 h. These cultures were also supplemented with morpholinopropane sulfonic acid buffer (0.1 M) to avoid pH fluctuations. Within this range, phosphate remained in excess in all cultures and varying the phosphate levels had little effect on the growth or jadomycin B titers (20–24 μg ml^{-1}, maximum).

Effect of carbon source on jadomycin B production

The effect of altering the concentration of galactose was examined in a production medium containing 10 mM-

phosphate and 30 mM-isoleucine supplemented with ethanol (6%, v/v) at 6 h post inoculation. Biomass accumulation and jadomycin B levels were measured over a 72 h period with maximum values being obtained by 48 h. An increase in D-galactose concentration from 1% (w/v) to 4% (w/v) was associated with increased jadomycin B titers and biomass accumulation (from 1.4 mg ml^{-1} to 2.4 mg ml^{-1}, dry weight) but an overall decrease in specific jadomycin B ($\mu g\ ml^{-1}$ dry weight) production (Fig. 4). Galactose remained in excess in all of the above cultures for a 72 h period but was exhausted after 48 h in cultures initially receiving only 0.5% (w/v). In the latter case, this resulted in diminished biomass (1.1 mg ml^{-1}) and jadomycin B (15 $\mu g\ ml^{-1}$) production. The nature of the carbon source was found to have some effect on jadomycin B titers with comparable concentrations resulting when either starch, glucose or maltose (2%, w/v) replaced galactose in the medium. Poor production (less than 15 $\mu g\ ml^{-1}$) resulted when either lactose or sucrose replaced galactose.

Effect of nitrogen source on jadomycin B production

The effect on jadomycin B production of L-isoleucine was examined in production medium containing 10 mM-phosphate and 3% (w/v) D-galactose supplemented with ethanol (6% v/v) at 6 h post inoculation. Biomass accumulation and jadomycin B levels were measured over a 72 h period with maximum values being obtained by 48 h. L-Isoleucine concentrations of 15, 30, 45, 60, and 75 mM were associated with increased final jadomycin B titers of 29, 45, 63, 66, and 69 $\mu g\ ml^{-1}$, respectively. Final biomass accumulation was unaffected by alteration of the L-isoleucine concentration within this range. In addition to the concentration, the nature of the nitrogen source was also found to be important. When alternative amino acids (30 mM) replaced L-isoleucine as the sole nitrogen source in the jadomycin production medium, ethanol-treated cultures produced a series of varied colored products (Table 1) suggesting the possibility that modified jadomycin B derivatives had been produced.

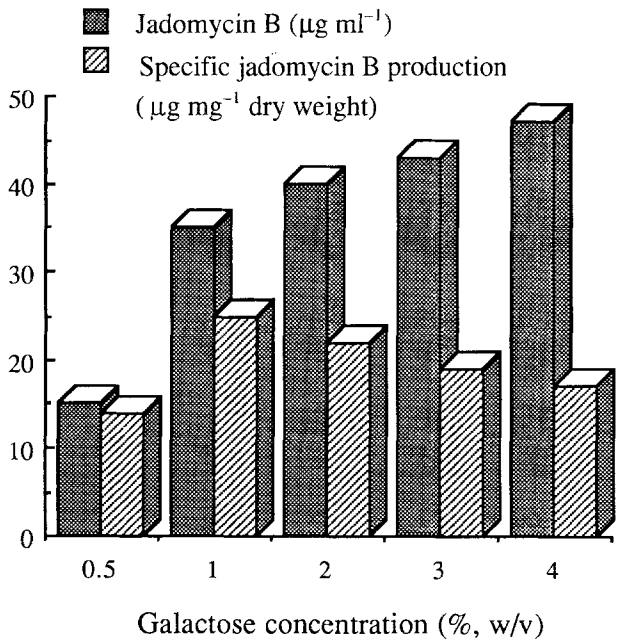

Fig. 4. Effect of varying the D-galactose concentration on production of jadomycin B in cultures of *Streptomyces venezuelae* growing in D-galactose-L-isoleucine medium supplemented with ethanol (6%, v/v) at 6 h post inoculation. Values shown are the maximum titers obtained (48 h post inoculation).

Effect of phage infection on jadomycin B production

When phage SV1 was added to D-galactose-L-isoleucine cultures 2 h after inoculation with *S. venezuelae* spores, increased orange pigmentation compared with controls was noted after 48 h growth at 30 °C; by HPLC analysis the phage infected cultures had accumulated 23 $\mu g\ ml^{-1}$ jadomycin B. This was accompanied by extensive cell lysis. Increased pigmentation was also observed following phage SV1 infection of mycelium on solid medium prepared by adding agar (16 g L^{-1}), starch (15 g L^{-1}) and yeast extract (2 g L^{-1}) to the D-galactose-L-isoleucine medium. When the pigment was obtained by dispersing the agar in distilled water (acidified to pH 3 with concentrated HCl), filtering, and extracting with ethyl acetate, it was shown to be jadomycin B by HPLC analysis. No pigmentation was seen on uninfected control plates.

Other known heat-shock-protein-inducing treatments examined such as addition of 25 μM-$CdCl_2$ [15] or either 100 μM or 200 μM-H_2O_2 [11] to D-galactose-L-isoleucine cultures at 6 h were ineffective in promoting jadomycin B production.

Effect of heat shock and ethanol treatment on chloramphenicol production

To investigate whether production of chloramphenicol, the other known antibiotic produced by *S. venezuelae* ISP5230, was initiated by jadomycin B-inducing conditions, chloramphenicol production was monitored in control, heat-shocked, and ethanol-treated cultures growing in D-galactose-L-isoleucine medium. Heat shocking appeared to have little effect on chloramphenicol production, titers being 0 $\mu g\ ml^{-1}$, 8 $\mu g\ ml^{-1}$ and 24 $\mu g\ ml^{-1}$ on days 2, 4 and 6, respectively, in the control cultures, and 0 $\mu g\ ml^{-1}$, 11 $\mu g\ ml^{-1}$ and 24 $\mu g\ ml^{-1}$ on days 2, 4 and 6, respectively, in the heat-shocked cultures. Ethanol-treated cultures (7 h post inoculation) initially at pH 6.5, 7, 7.5, or 8 accumulated no chloramphenicol; cultures initially at pH 6 accumulated a small amount (3 $\mu g\ ml^{-1}$) by day 4.

DISCUSSION

Of the variety of treatments known to induce a heat-shock response in prokaryotic cells, heat shock, ethanol exposure, and phage infection are associated with the production of jadomycin B in cultures of *S. venezuelae* ISP5230 growing at 27 °C. In the absence of these physiological stress conditions, very little jadomycin B is produced. This accounts for the failure to detect jadomycin compounds

TABLE 1

Effect of varying the L-amino acid nitrogen source on the production of jadomycin B-derived pigments in cultures of *Streptomyces venezuelae* supplemented with ethanol (6%, v/v)

	Color of culture medium				
	Black	Orange	Purple	Red	Yellow
Nitrogen source	Tryptophan	Alanine Glutamic acid Isoleucine Leucine Methionine Serine Valine	Threonine	Glycine Lysine Phenylalanine Tryosine	Arginine Asparagine Aspartic acid Cysteine Glutamine Histidine

during growth of *S. venezuelae* ISP5230 under chloramphenicol production conditions. The two antibiotics appear to have very different regulatory controls as no chloramphenicol was detected in jadomycin-producing cultures supplemented with ethanol nor was chloramphenicol production enhanced in heat-shocked cultures.

An interesting feature of both heat shock and ethanol exposure was that the timing of the treatment following inoculation into D-galactose-L-isoleucine medium was critical. Treatments after 17 h post inoculation were ineffective in initiating jadomycin B production. Perhaps significantly, the level of heat-shock protein induction following heat stress has been frequently reported to depend on the growth or development phase [16]. If the initiation of jadomycin B production is linked to the heat-shock response, a similar association might be expected.

Although the mechanism whereby ethanol treatment induces the production of jadomycin B has not been determined, we suggest that it relates to the demonstrated ability of this treatment to trigger a heat-shock response and we are currently investigating this possibility. Alternatively, ethanol could be serving as a source of polyketide precursors, (although sodium acetate added at the same concentration did not induce jadomycin B production), or to increase membrane permeability and thus promote release of the metabolites.

In the presence of ethanol (6%, v/v), jadomycin B production was insensitive to alterations in the concentrations of phosphate in the production medium. This is not surprising as growth is severely restricted following the addition of ethanol (an increase in biomass from ~0.7 mg ml^{-1} dry weight, at the time of ethanol addition, to ~2 mg ml^{-1} by 48 h; compared with 5 mg ml^{-1} at 48 h in unsupplemented control cultures [6]) and phosphate at the concentrations indicated does not become depleted from the media. It seems likely that more subtle nutritional effects on production are being obscured under these severely growth-limiting conditions.

Increased growth and decreased specific jadomycin B biosynthesis in the presence of higher levels of D-galactose, however, suggests that jadomycin B production may be under some form of growth rate control. A similar situation, where production is related to a suboptimal rate of growth, has been described for numerous other antibiotics [5]. An increase in jadomycin B titers was also observed with an increase in L-isoleucine concentration in the medium although no corresponding increase in biomass was observed. This would be explained by our prediction that L-isoleucine is incorporated directly into the jadomycin B precursor molecule during biosynthesis. In support of this hypothesis, we observed that the color of the pigmented product changed when various other amino acids were substituted for L-isoleucine. We predict that when other amino acids are provided in the growth medium, they will be incorporated in place of L-isoleucine producing new jadomycin analogs. This has been confirmed in the case of cultures grown on glycine which produce a product (jadomycin C) formed from the incorporation of glycine into the jadomycin precursor backbone (S.W. Ayer et al., unpublished results). This appears to be an excellent system of directing biosynthesis of jadomycin analogs through simple alterations of the amino acid content of the production medium. The structures and bioactivities of L-leucine- and L-tryptophan-derived jadomycin analogs are currently being studied.

Viral infection is known to induce several heat-shock genes in various cell types. In *Escherichia coli* for example, phage lambda induces synthesis of GroEL and DnaKJ within minutes of infection [7]. The association of increased synthesis of jadomycin B with SV1 infection is perhaps attributable again to induction of a heat-shock response.

In conclusion, media conditions for the optimal production of the pigmented antibiotic, jadomycin B, by *S. venezuelae* ISP5230 were defined. Highest titers are obtained in ethanol-treated cultures where jadomycin B concentrations are associated with increases in the D-galactose and L-isoleucine content of the medium. Induction of jadomycin B production only occurs following ethanol addition during the first 17 h of growth. Substitution of other amino acids for L-isoleucine as the sole nitrogen source results in the production of a variety of pigmented products and possibly represents a

method of directing the biosynthesis of jadomycin B analogs. The general applicability of high temperatures and ethanol treatments as a means of inducing the production of antibiotics by other *Streptomyces* is currently being investigated.

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