The Neocarzinostatin Biosynthetic Gene Cluster from *Streptomyces carzinostaticus* ATCC 15944 Involving Two Iterative Type I Polyketide Synthases

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Summary

The biosynthetic gene cluster for the enediyne antitumor antibiotic neocarzinostatin (NCS) was localized to 130 kb continuous DNA from *Streptomyces carzinostaticus* ATCC 15944 and confirmed by gene inactivation. DNA sequence analysis of 92 kb of the cloned region revealed 68 open reading frames (ORFs), 47 of which were determined to constitute the NCS cluster. Sequence analysis of the genes within the NCS cluster suggested dNDP-D-mannose as a precursor for the deoxy aminosugar, revealed two distinct type I polyketide synthases (PKSs), and supported a convergent model for NCS chromophore biosynthesis from the deoxy aminosugar, naphthoic acid, and enediyne core building blocks. These findings shed light into deoxysugar biosynthesis, further support the iterative type I PKS paradigm for enediyne core biosynthesis, and unveil a mechanism for microbial polycyclic aromatic polyketide biosynthesis by an iterative type I PKS.

Introduction

The enediyynes are a class of natural products that consist of a unique molecular architecture and exhibit phenomenal antitumor activity [1–4]. They are structurally characterized by an enediyne core containing two acetylenic groups conjugated to a double bond or incipient double bond within a 9-membered ring, such as neocarzinostatin (NCS) and C-1027, or a 10-membered ring, such as calicheamicin (CAL) (Figure 1). The enediyne cores are further decorated with a variety of peripheral moieties, and the 9-membered ring enediynes, also known as chromopeptides, are noncovalently associated with an apo-protein that has been proposed to dually stabilize and transport the bioactive chromophore.

As a family, the enediyynes share a common mechanism of action: the chromophore undergoes electronic rearrangement to form a diradical species, which subsequently can abstract hydrogen atoms from the deoxyribose of DNA leading to single- and double-stranded DNA lesions [3, 4]. A thiol is required to activate radical formation, although a few enediyynes have been reported to form diradicals in a thiol-independent manner, likely the result of the inherent instability of the 9-membered ring enediyne core. The ability of the enediyynes to ultimately cleave DNA has attracted great interest in developing these compounds into anticancer drugs. A CD33 mAB-CAL conjugate (Mylotarg) was approved in the US to treat acute myeloid leukemia in 2000 [5]. A poly(ethylene-co-maleic acid)-NCS conjugate (SMANCS) was approved in Japan and has been marketed for use against hepatoma since 1994 [2]. Several antihepatoma mAB-C-1027 conjugates have displayed high tumor specificity and exerted a strong inhibitory effect on the growth of established tumor xenografts [6]. In general, however, the high cell toxicity of the enediyynes has limited their clinical utility, and ways of harnessing the potent activity of the enediyynes for anticancer drug development is currently an ongoing area of intense research [1–6].

NCS, produced by *Streptomyces carzinostaticus* ATCC 15944, was the first member of the enediyne family to be structurally elucidated [7]. Consequently, NCS has been widely used as a model for the enediyynes to investigate the molecular details of chromophore activation and DNA binding and cleavage [1–3]. The structure of the NCS chromoprotein has been solved by 1H and 13C NMR spectroscopy [8, 9] and X-ray crystallography [2]. NCS chromophore-DNA complexes have also been well studied, revealing multiple modes of DNA recognition and binding [10].

Biosynthetic studies on the enediyne family have been limited primarily by their extreme instability and low production in fermentation, rendering it difficult to carry out in vivo feeding experiments [4, 11]. In spite of the difficulties, the enediyne cores of NCS [12], dynemicin [13], and esperamicin [14] were established to originate from a minimum of eight head-to-tail acetate units, but it was unclear until recently whether this assembly occurred by polyketide biosynthesis or degradation of a fatty acid precursor. The recent cloning, sequencing, and characterization of the C-1027 [15] and CAL [16] biosynthetic gene clusters revealed a single enediyne polyketide synthase (PKS) gene, establishing a polyketide paradigm for enediyne biosynthesis [7–19]. Also found within the C-1027 gene cluster were *cagA*, encoding the C-1027 apo-protein, and the biosynthetic machinery for the peripheral moieties, suggesting a convergent strategy for chromoprotein enediyne biosynthesis [15].

We proposed that NCS is biosynthesized in a similar fashion as C-1027 on the basis that NCS and C-1027 share a similar enediyne core, a 6-deoxysugar moiety, and an apo-protein, and therefore adopted the same strategy as that for C-1027 to clone the NCS biosynthetic gene cluster. Here we report the cloning and
identification of the NCS biosynthetic gene cluster from S. carzinostaticus ATCC15944, the sequence of the complete NCS gene cluster, determination of the cluster boundaries, and functional assignments of the gene products. On the basis of these results, we propose a convergent model for NCS chromophore biosynthesis from the deoxy aminosugar, naphthoic acid, and enediyne core building blocks. The NCS cluster is characterized with two distinct type I PKSs, NcsE for the enediyne core and NcsB for the naphthoic acid moiety, and a deoxysugar biosynthetic pathway most likely starting from dNDP-D-mannose. These findings further support the iterative type I PKS paradigm for enediyne core biosynthesis [15–19], unveil a novel mechanism for microbial polycyclic aromatic polyketide biosynthesis by an iterative type I PKS [19, 20], and shed new light into deoxysugar biosynthesis. The NCS cluster, together with the growing list of other enediyne biosynthetic gene clusters [15–18], provides a unique opportunity to investigate the molecular basis of enediyne biosynthesis by a comparative genomics approach.

Results and Discussion

Identification, Localization, and Cloning of the \textit{ncs} Gene Cluster from \textit{S. carzinostaticus} ATCC15944

Deoxysugars are frequently found in secondary metabolites and are vital components for the efficacy and specificity of a natural product’s biological activity. The biosynthetic pathways of these unusual sugars have been extensively investigated. The first committed step of the biosynthesis of all deoxyhexoses is through the intermediate dNDP-4-keto-6-deoxyhexose, a reaction catalyzed by an NAD$^+$-dependent oxidoreductase from the precursor dNDP-D-hexose (most commonly dNDP-D-glucose, and hence the name of NGDH derived from dNDP-D-glucose 4,6-dehydratase) [21]. We have previously taken advantage of the highly conserved nature of NGDHs and used degenerate primers [22] to amplify the NGDH gene \textit{sgcA} by PCR from 	extit{S. globisporus} and this locus was used as a starting point for chromosomal walking leading to the eventual localization of the entire C-1027 biosynthetic gene cluster [15, 23]. NCS contains a similar 6-deoxyhexose to C-1027, and as a result, we adopted the same strategy to identify the \textit{ncs} gene cluster. A distinct product with the predicted size of 550 bp was amplified by PCR and confirmed to be a putative NGDH gene, serving as the first probe (Figure S1A).

The cloning of the genes necessary for C-1027 biosynthesis revealed that \textit{cagA}, encoding the C-1027 apo-protein, was within the boundaries of the gene cluster [15]. Therefore, the gene for the NCS apo-protein, \textit{ncsA}, was used as the second probe. The primary sequence for \textit{ncsA} has previously been established [reviewed in 11], and PCR with primers designed according to the known sequence yielded a distinct product with the predicted size of 590 bp. The PCR product was cloned into pGEM-T to yield pBS5002, confirmed by DNA sequencing, and utilized as the second probe.

The digoxigenin-[DIG]-labeled NGDH and \textit{ncsA} probes were both used to screen approximately 4800 clones of the genomic library. While the NGDH probe resulted in the isolation of four overlapping cosmids spanning 67 kb (Figure S1B), the \textit{ncsA} probe afforded fourteen overlapping cosmids spanning 64 kb, as represented by pBS5002, pBS5003, and pBS5004 (Figure 2A). However, the two loci do not overlap. Since genes for antibiotic production are known to cluster in one region of the chromosome in \textit{Streptomyces}, we set out to first determine if the putative NGDH locus was required for NCS biosynthesis by gene disruption. Surprisingly, inactivation of the NGDH gene has no effect on NCS production, excluding its involvement in NCS biosynthesis (Figure S1C).

We next turned our attention to the \textit{ncsA} locus. Since it was proposed that \textit{ncsA} should reside within the NCS gene cluster, a 7.5 kb BglII fragment containing \textit{ncsA} was cloned from pBS5004. DNA sequence analysis of this fragment revealed six complete ORFs (including \textit{ncsA}) and one incomplete ORF. Remarkably, the four ORFs encode a dNDP-D-mannose synthase (NcsC), dNDP-hexose 4,6-dehydratase (NcsC1, a second distinct NGDH gene in this organism), N-methyltransferase (NcsC5), and glycosyltransferase (NcsC6). These are the enzymes that would be predicted to be essential for biosynthesis of the deoxy aminosugar moiety of NCS chromophore (Figure 3A), indicating that the NCS biosynthetic locus was identified. To ensure that we have full coverage of the entire NCS gene cluster, additional chromosomal walking from the left end of pBS5002 (probe 3) and the right end of pBS5004 (probe 4) was carried out, leading to the eventual localization of a 130 kb continuous DNA region covered by overlapping cosmids as exemplified by pBS5002, pBS5003, pBS5004, pBS5005, pBS5007, pBS5010, pBS5013, pBS5015, and pBS5017 (Figure 2A).

Sequencing and Organization of the \textit{ncs} Gene Cluster

Three representative overlapping cosmids, pBS5002, pBS5004, and pBS5017 were selected for DNA se-
Neocarzinostatin Biosynthetic Gene Cluster

Figure 2. S. carzinostaticus ATCC15944 as Represented by Nine Cosmid Clones and Restriction Map and Genetic Organization of the ncs Biosynthetic Gene Cluster

(A) A 140 kb locus from S. carzinostaticus ATCC15944 as represented by nine cosmid clones. Solid black bars indicate regions whose sequence has been determined (AY117439).

(B) Restriction map and genetic organization of the ncs biosynthetic gene cluster. Proposed functions for individual orfs are shade-coded and summarized in Table 1.

quencing by a shotgun method, revealing 68 ORFs (Figure 2B). The overall GC content of the sequenced region was 68.88%, slightly lower than the characteristic ~75% for Streptomyces DNA [22]. The sequence was analyzed by comparison to database sequences, and functional assignments to individual ORFs are deposited in the GenBank under accession number AY117439 and summarized in Table 1. Consistent with the structure of NCS, those identified within the ncs cluster include fourteen genes encoding the enediyne core biosynthesis, seven genes encoding deoxy aminosugar biosynthesis, four genes encoding naphthoic acid biosynthesis (Figure 3), as well as seven ORFs encoding putative regulation proteins (NcsR1 to NcsR7), two ORFs encoding resistance proteins (NcsA and NcsA1), and twelve ORFs whose functions could not be predicted or assigned on the basis of sequence analysis (ORFs 13, 14, 19, 24, 27, 29, 32, 34, 49, 55, 56, and 57) (Figure 2B).

ncs Cluster Boundaries

To determine the boundaries of the ncs cluster, orf6 and ncsR1 were inactivated by gene disruption, and the resultant mutant strains SB5004 (orf6) (Figures 4A and 4B) and SB5005 (JncsR1) (Figures 4C and 4D) were confirmed by Southern analysis. orf6 encodes a putative membrane protein, and the orf6 mutant strain SB5004 retained its NCS productivity similar to the wild-type strain based on bioassay (Figure 4E, lane 2 versus lane 1), suggesting that it is outside of the ncs cluster. In contrast, ncsR1 encodes a putative γ-butyro-lactone biosynthesis enzyme, and the JncsR1 mutant strain SB5005 completely lost its ability to produce NCS, confirming that ncsR1 is essential for NCS production (Figure 4E, lane 3 versus lane 1). These results, together with the functional assignment of NCS gene products based on primary sequence analysis (Table 1), as well as organizational comparisons to the gene cluster for C-1027 [23], support the conclusion that the ncs cluster is minimally contained within the region from ncsC4 to orf57, which consists of a total of 47 ORFs spanning 63 kb (Figure 2B). The availability of genes within the cluster boundaries provides a basis to propose the NCS biosynthetic pathway.

Biosynthesis of the Deoxy Aminosugar Moiety

Seven genes, ncsC to ncsC6, encode proteins that are homologous to enzymes involved in deoxysugar biosynthesis (Table 1), and accordingly, the biosynthesis of the deoxy aminosugar moiety is proposed as outlined in Figure 3A. The deduced product of ncsC resembles a family of dNDP-D-mannose synthases, suggesting the deoxy aminosugar moiety is derived from D-mannose-1-phosphate. After dNDP-activation of mannose, NcsC1, which is similar to dNDP-hexose dehydratases, catalyzes the formation of dNDP-4-keto-6-deoxyhexose. NcsC2, which is similar to dNDP-hexose dehydratases, catalyzes the 2,3 dehydration of dNDP-hexose derivative to form an α,β-unsaturated-4-ketosugar. NcsC3, similar to a variety of histidine or phenylalanine ammonia lyases and the aminomutase SgcC4 in C-1027 biosynthesis, incorporates an amino group at C-2. NcsC4, a putative
Figure 3. Proposed Biosynthetic Pathway for NCS Chromophore
(A) Deoxy aminosugar; (B) naphthoic acid; (C) the enediyne core and a model for convergent assembly from the three building blocks.

D-Man-P, D-mannose-1-phosphate; D-Glu-P, D-glucose-1-phosphate; "NH₃," a yet to be established amino donor; and HMNA, 2-hydroxy-5-methoxy-1-naphthoic acid.

dNDP-hexose 4-ketoreductase, epimerizes the hydroxyl group at C-4 by reduction of dNDP-2-amino-4-keto-6-deoxyhexose. NcsC5, related to various N-methyltransferases in deoxy aminosugar biosynthesis, methylates the primary amine (Figure 3A, path a). The final gene product, NcsC6, encoding a putative glycosyl transferase, is responsible for transferring the deoxy aminosugar moiety to the enediyne core (Figure 3C).

We have proposed, on the basis of sequence homology of NcsC to dNDP-D-mannose synthases, that biosynthesis of the sugar moiety begins with dNDP-D-mannose. This is unusual, since most of the deoxysugar biosynthetic pathways characterized to date for secondary metabolites start from dNDP-D-glucose [21]. We are aware of only three examples in Streptomyces in which dNDP-D-glucose was implicated as the precursor for deoxysugar biosynthesis [24–26]. Since the C-2 hydroxyl group is ultimately eliminated in the deoxy aminosugar moiety of NCS, it cannot be excluded that the synthesis begins with D-glucose-1-phosphate and that NcsC is instead a dNDP-D-glucose synthase (Figure 3A, path b). However, it is evident upon sequence alignment that the C-terminal region of NcsC1 has low sequence homology to other known NGDHs (Figure S1A). The latter not only explains our inability to amplify NcsC1 by the PCR method using primers designed according to dNDP-glucose 4,6-dehydratase enzymes from Streptomyces [22, 23], but also agrees with the proposal that dNDP-D-mannose, instead of dNDP-D-glucose, is the most likely substrate for NcsC1.

The amino transferase reaction catalyzed by NcsC3 is supported by our recent characterization of SgcC4 for C-1027 biosynthesis [27, 28]. SgcC4, although clearly related to ammonia lyases, was shown to be an aminotransferase with preferred intramolecular amino transfer following the formation of an α,β-unsaturated carboxylic acid intermediate; however, the Michael addition of an exogenous amino group to this intermediate was also detected [28]. The latter activity is reminiscent to what is proposed for NcsC3. Furthermore, histidine and phenylalanine lyases contain a critical serine in a highly conserved A-S-G motif that is used to form the 4-methylimidazole-5-one prosthetic group. While exhibiting high overall amino acid sequence homology to SgcC4 and other ammonia lyases, NcsC3 lacks the conserved serine residue, hence cannot be functional as a lyase, a fact that is consistent with NcsC3 as an aminotransferase.

Biosynthesis of the Naphthoic Acid Moiety
Four genes, ncsB, ncsB1, ncsB2, and ncsB3 were identified whose deduced functions (Table 1) would support
their involvement in the biosynthesis of the naphthoic acid moiety of NCS as outlined in Figure 3B. Previous biosynthetic studies on the naphthoic acid moiety revealed that the C12 naphthoic ring is derived from a hexaketide by six head-to-tail condensations of acetate, and the O-methyl group is derived from S-adenosyl-L-methionine (AdoMet) [12]. While the current paradigm for the biosynthesis of an aromatic polyketide such as the naphthoic acid moiety in bacteria calls for an iterative type II PKS [19], no candidate genes encoding type II PKS could be found within the NCS cluster. Instead, the NCS gene cluster contained two type I PKSs, NcsE and NcsB. NcsE is clearly related to CalE8 [16] and SgcE [15], the PKSs responsible for the biosynthesis of the enediyne core. NcsB, in contrast, shows head-to-tail homology (excluding the keto-reductase [KR] domain) to AvIM [29] and CalO5 [16], both of which are iterative type I PKSs proposed to catalyze the biosynthesis of an orsellinic acid moiety for avilamycin in S. viridochromogenes and CAL in Micromonospora echinospora, respectively (Figure 5A). NcsB consists of characteristic domains for type I PKS, including a ketosynthase (KS), acyltransferase (AT), dehydratase (DH), KR, and acyl carrier protein (ACP). It catalyzes the formation of a nascent linear hexaketide intermediate then undergoes an
intramolecular aldol condensation to furnish the naphthoic acid structure. Subsequent hydroxylation at C-7 followed by O-methylation affords the fully modified naphthoic acid moiety, and NcsB3, a homolog of the P-450 family of hydroxylases, and NcsB1, a member of the AdoMet-dependent methyltransferase family of enzymes, serve as candidates for these two steps (Figure 3B). It remains to be established if the free naphthoic acid or the naphthoyl-S-NcsB is the preferred substrate for NcsB3 and NcsB1. Should the latter be the case, ncsB2, located just upstream of ncsB, encodes a putative CoA ligase, serves as a candidate to catalyze the attachment of naphthoyl moiety from naphthoyl-S-NcsB to the enediyne core (Figure 3C). The proposed activity of NcsB2 would resemble the catalysis performed by CoA ligases during the synthesis of CoA ester from carboxylic acid but in the reverse direction in the presence of a donor hydroxyl group.

Until recently, aromatic polyketides in bacteria were believed to be biosynthesized solely by type II PKSs [19]. Therefore, early attempts to clone the NCS gene cluster utilized type II PKS genes as probes, and the finding of NcsB as a type I PKS explained why these experiments failed. Currently, NcsB represents a third example of an iterative type I PKS for the biosynthesis of an aromatic polyketide, and is the first to our knowledge to extend this emerging paradigm from monocyclic to include polycyclic aromatic compounds. This has indeed been confirmed recently by Sohng and coworkers who isolated 2-hydroxy-5-methyl-1-naphthoic acid, thenaphthoic acid moiety, and NcsB3, a homolog of the P-450 family of hydroxylases, and NcsB1, a member of the AdoMet-dependent methyltransferase family of enzymes, serve as candidates for these two steps (Figure 3B). It remains to be established if the free naphthoic acid or the naphthoyl-S-NcsB is the preferred substrate for NcsB3 and NcsB1. Should the latter be the case, ncsB2, located just upstream of ncsB, encodes a putative CoA ligase, serves as a candidate to catalyze the attachment of naphthoyl moiety from naphthoyl-S-NcsB to the enediyne core (Figure 3C). The proposed activity of NcsB2 would resemble the catalysis performed by CoA ligases during the synthesis of CoA ester from carboxylic acid but in the reverse direction in the presence of a donor hydroxyl group.

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Biosynthesis of the Eneidyne Core
At least fourteen genes, ncsE to ncsE11 and ncsF1 to ncsF2, could be identified within the ncs cluster, the deduced functions of which would support their roles in the NCS enediyne core biosynthesis as outlined in Figure 3C. The enediyne core was previously predicted to be synthesized by an iterative type I PKS with five domains, of which the KS, AT, KR, and DH are characteristic of known type I PKSs [15–19]. NcsE shows head-to-tail sequence homology to the SgcE [15] and CalE8 [16] enediyne PKSs (Figure 5B). Consequently, we propose that NcsE, in a mechanistic analogy to other enediyne PKSs, catalyzes the formation of the naphthoic acid structure. Subsequent hydroxylation at C-7 followed by O-methylation affords the fully modified naphthoic acid moiety, and NcsB3, a homolog of the P-450 family of hydroxylases, and NcsB1, a member of the AdoMet-dependent methyltransferase family of enzymes, serve as candidates for these two steps (Figure 3B). It remains to be established if the free naphthoic acid or the naphthoyl-S-NcsB is the preferred substrate for NcsB3 and NcsB1. Should the latter be the case, ncsB2, located just upstream of ncsB, encodes a putative CoA ligase, serves as a candidate to catalyze the attachment of naphthoyl moiety from naphthoyl-S-NcsB to the enediyne core (Figure 3C). The proposed activity of NcsB2 would resemble the catalysis performed by CoA ligases during the synthesis of CoA ester from carboxylic acid but in the reverse direction in the presence of a donor hydroxyl group.

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A Convergent Strategy for NCS Assembly

A convergent strategy could be envisaged for the assembly of the NCS chromophore from the three individual building blocks of the deoxyaminosugar, naphthoic acid, and enediyne core (Figure 3C). While the coupling between dNDP-sugar and the enediyne core is catalyzed by the NcsC6 glycosyltransferase, that between naphthoyl-S-NcsB and the enediyne core is most likely catalyzed by the NcsB2 CoA ligase. Although the cyclic carbonyl carbon of NCS has previously been shown to originate from carbonate [12], no obvious candidate catalyzing the attachment of carbonate could be identified within the gene cluster. The convergent biosynthetic strategy for NCS once again underscores nature’s efficiency and versatility in synthesizing complex molecules.

Significance

NCS was the first member of the enediyne family of antitumor antibiotics to be structurally elucidated, yet cloning and characterization of the NCS biosynthetic gene cluster has heretofore met with little success in spite of considerable effort. Early cloning strategies were based on the general assumption that the biosynthesis of the naphthoic acid moiety would be catalyzed by a type II PKS, of the enediyne core by either a type I or type II PKS, and of the deoxyaminosugar from dNDP-D-glucose, none of which turned out to be correct or unique to the NCS pathway. The presence
of three copies of the NCS biosynthetic gene cluster in *S. carzinostaticus* ATCC15944 also explains why all early attempts in disrupting the NCS pathway yielded ambiguous results. Here we report the cloning and confirmation of the NCS biosynthetic gene cluster from *S. carzinostaticus* ATCC15944 and demonstrate the feasibility of manipulating NCS biosynthesis in vivo gene inactivation. Sequence analysis of the genes within the NCS cluster suggested dNDP-D-mannose as a precursor for the deoxy aminosugar, revealed two distinct type I polyketide synthases (PKSs), NcsE for the enediyne core and NcsB for the naphthoic acid moiety, and supported a convergent model for NCS chromophore biosynthesis from the deoxy aminosugar, naphthoic acid, and enediyne core building blocks. These findings shed light into deoxyxsugar biosynthesis, further support the iterative type I PKS paradigm for enediyne core biosynthesis, and unveil a mechanism for microbial poly cyclic aromatic polyketide biosynthesis by an iterative type I PKS. The NCS cluster, together with the growing list of other enediyne biosynthetic gene clusters, provide a unique opportunity to investigate the molecular basis of enediyne biosynthesis by a comparative genomics approach and to apply combinatorial bio-synthetic methods to the enediyne biosynthetic machinery for anticancer drug discovery and development.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Reagents**

Bacterial strains and plasmids used in this study are summarized in Table S1. Biochemicals, chemicals, media, restriction enzymes, and other molecular biology reagents were from standard commercial sources.

**DNA Isolation, Manipulation, and Sequencing**

DNA isolation and manipulation in *E. coli* [30] and *Streptomyces* [31] were carried out according to standard methods. For Southern analysis, DIG labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Roche Applied Science, Indianapolis, IN). PCR amplifications were carried out on a GeneAmp 2400 thermocycler (Perkin-Elmer/ABI, Foster City, CA) using either Vent DNA polymerase (New England Biolabs, Beverly, MA) or TaKaRa La-Taq DNA polymerase (Takara Mirus Bio Inc., Madison, WI). For NGDH, the following pair of degenerate primers was used: 5′-CSC GGS GSS GCS GGS TTC ATC GG-3′ (forward) and 5′-GGW GWR CTG GYR SGG SCC GTA GTT G-3′ (reverse) (R, A+G; S, C+G; W, A+T; Y, C+T) and for ncsA, the following pair of primers was used: 5′-GCT CCG GCG GCT TCT TCA G-3′ (forward) and 5′-GCG GAG GCG CAT CTC GTA TG-3′ (reverse). Primer synthesis and DNA sequencing were performed at the Biotechnology Center, University of Wisconsin-Madison.

**Genomic Library Construction and Screening**

A genomic library of *S. carzinostaticus* ATCC15944 was constructed in pGJ446 according to standard protocols [31]. *E. coli* XL1-Blue MRF' and Gigapack III XL packaging extract (Stratagene, La Jolla, CA) were used for library construction according to manufacturers’ instructions. The genomic library (4800 colonies) was screened by colony hybridization with the PCR-amplified NGDH or ncsA fragment as a probe, respectively, and the resultant positive clones were further confirmed by Southern hybridization.

**Gene Inactivation and Complementation**

We have recently found that *S. carzinostaticus* ATCC15944 contains three copies of the NCS biosynthetic gene cluster that are identical within the sequenced 92 kb DNA region (K.N., J.Z., and B.S., unpublished data). This complicates any attempt to disrupt the NCS pathway since total abolishment of NCS production requires the inactivation of all three copies of the NCS cluster. To identify the correct mutant, we screened a large pool of recombinant isolates with the desired antibiotic phenotype by Southern analysis. This pool includes mutants with one, two, or all three copies of the NCS cluster disrupted, whose genotypes can be distinguished upon Southern analysis. For example, if only one copy is inactivated, Southern analysis will show a genotype as if it were a mixture of the expected mutant (the inactivated copy) and the wild-type (the other two intact copies). In contrast, if all three copies of the cluster are inactivated, Southern analysis will yield a distinct pattern such as that shown in Figures 4 and 6. Only the mutants whose genotypes were confirmed to result from inactivation of all three copies of the NCS cluster were selected for further study.

Introduction of plasmid DNA into *S. carzinostaticus* was carried out by polyethylene glycol-(PEG)-mediated protoplast transformation, following standard procedure [31] with minor modifications. In brief, mycelia obtained from YEME culture supplemented with 25 mM MgCl2 were used to prepare *S. carzinostaticus* protoplasts, and upon mixing with plasmid DNA, the transformed protoplasts were spread onto R2YMP (R2YE enriched with 0.5% of Difco malt extract and bacto peptone) plates for regeneration. After incubation at 28°C for 20 hr, the plates were overlaid with soft R2YMP supplemented with the appropriate antibiotic and incubation continued until colonies appeared (5-7 days).

To inactivate orf6, an internal fragment of orf6 was amplified by PCR using the following primers 5′-AGG CTG TGC TGC AG-3′/5′-TGG ACC TGC TGC TCA CC-3′. The PCR product was cloned directly into pGEM-T to yield pBS5025. After sequencing to confirm PCR fidelity, a 0.80 kb PstI-BamHI fragment was recovered from pBS5025 and cloned into the same sites of pOJ260 to afford the gene disruption construct pBS5021 (Figure 4A).

To inactivate ncsR1, an internal fragment of ncsR1 was amplified by PCR using the following pair of 5′-CCG GAT CCC GCT CAC AGC TTC TAC AGC-3′/5′-GAA ATT CCG TCA TTT GAG TGA CGG-3′ (the PCR incorporated BamHI and EcoRI sites are underlined). The PCR product was directly cloned into pGEM-T to yield pBS5026. After sequencing to confirm PCR fidelity, a 0.96 kb BamHI-EcoRI fragment was recovered from pBS5026 and cloned into the same site of pOJ260 to afford the gene disruption construct pBS5022 (Figure 4C).

pBS5021 or pBS5022 was transformed into *S. carzinostaticus* ATCC15944 by PEG-mediated protoplast transformation, and colonies that were apramycin resistant were identified as gene disruption mutant strains. Thus, introduction of pBS5021 or pBS5022 resulted in the isolation of the SB5004 (orf6) or SB5005 (ncsR1) mutant strains, respectively, in which orf6 or ncsR1 has been disrupted by the insertion of the pOJ260 vector into the middle of the reading frame (Figures 4A and 4C). The genotype of SB5004 and SB5005 was confirmed by Southern analysis (Figures 4B and 4D). The SB5004 and SB5005 strains were then similarly cultured and analyzed for NCS production by bioassay with the ATCC15944 wild-type strain as a control (Figure 4E).

To inactivate ncsE, an 11 kb Ndel fragment (nucleotide no. 51689-62298) that contained the entire ncsE gene, was cloned from pBS5017 into the same sites of pGEM-S2Z to yield pBS5018. A 3.4 kb SphI-Nhel fragment and a 4.4 kb MluI-BglII fragment were isolated from pBS5018, and cotigated with the 1.8 kb Nhel-MluI fragment that contains the erythromycin resistance gene, ermE [31]. The product was digested with XbaI and BamHI and ligated into the similar sites of pOJ260 to yield pBS5019, in which a 1.2 kb internal Nhel-Mlu fragment of ncsE encoding the AT (partial), ACP (intact), and KR (partial) domains was replaced by ermE (Figure 6A). pBS5019 was introduced into *S. carzinostaticus* ATCC15944 by PEG-mediated protoplast transformation. Colonies that were apramycin sensitive and erythromycin resistant were identified as *S. carzinostaticus* SB5002 mutant whose genotype was further confirmed by Southern analysis (Figure 6B). To make an ncsE expression construct, a 450 bp EcoRI-BamHI fragment that harbored the *ermE* promoter from pWHM79 [32] and an 8.9 kb BglII-SphI fragment that contained the intact ncsE gene as well as its upstream ncsE5 and downstream ncsE10 genes from pBS5017 were cloned into the EcoRI-XbaI sites of pBS3031 [33] to yield pBS5020.
The latter was introduced into S. carzinostaticus SB5002 by PEG-mediated protoplast transformation to complement the ncsEermE mutation, yielding strain SB5003. Recombinant strains were cultured and analyzed for NCS production by bioassay and/or HPLC with the S. carzinostaticus wild-type strain as a control (Figures 6C and 6D).

The PCR-amplified NGDH locus was similarly inactivated, and the genotype and phenotype of the resultant mutant strain SB5001 (ΔNGDH) was similarly investigated (Figure S1).

Production, Isolation, and Analysis of NCS

S. carzinostaticus wild-type and recombinant strains were grown on ISP-2 or R2YP plates (with appropriate antibiotic for recombinant strains) at 28°C for sporulation [31]. For NCS production, 50 µl of spore suspension (cfu >10^8 cells/ml) of the S. carzinostaticus ATCC15944 wild-type or recombinant strains was inoculated into 50 ml of seed media (starch 2%, soybean flour 2%, NaCl 0.5%, Difco yeast extract 0.5%, CaCO3 0.2%, MnSO4•H2O 0.0005%, CuSO4•H2O 0.0005%, and ZnSO4•7H2O 0.0005% [pH 7.2]) in a 250 ml flask and incubated at 28°C and 250 rpm for 2 days. Seed culture (1 ml) was transferred into 15 ml of production media (4% glucose, 1.5% Difco casamino acid, MgSO4 1.25%, CaCO3 0.2%, and KH2PO4 0.1% [pH 7.2]) in a 250 ml flask and incubated at 28°C and 250 rpm for 2 to 4 days.

For NCS chromophore isolation, the fermentation culture was centrifuged to remove the mycelia, and the broth, upon adjustment to pH 3.5 with 1N HCl, was centrifuged again to remove any acid precipitate. The resultant supernatant was then fractionated by addition of (NH4)2SO4, and NCS chromoprotein was precipitated between 40%–85% saturation. The precipitated NCS chromoprotein was collected by centrifugation, dissolved in 5 volumes of 15 mM NaOAc (pH 4.5) buffer, and dialyzed against H2O at 4°C. The dialyzed sample was freeze-dried and NCS chromophore was extracted with MeOH. The isolated yield for the NCS chromophore from the wild-type strain by this method varies between 1–5 mg/l.

HPLC analysis of NCS chromophore was carried out on a Cadenza CD-C18 analytical column (3 µm, 150 x 4.6 mm, Intertek Corp., Kyoto, Japan). The column was equilibrated with 50% solvent A (MeOH/H2OAc [100:2]) and 50% solvent B (H2O/H2OAc [100:2]) and developed with the following program (0–15 min, 50% A/50%B; 15–25 min, a linear gradient from 50% A/50%B to 95% A/5%B; 25–30 min, constant 95% A/5%B) at a flow rate of 0.5 ml/min and UV detection at 320 nm using a Dynamic gradient HPLC system (Rainin Instruments, Oakland, CA). ESI-MS analysis of NCS was performed on an Agilent 1000 HPLC-MS SDL instrument (Agilent Technologies, Palo Alto, CA).

Bioassay of the NCS chromoprotein complex against Micrococcus luteus ATCC9431 was carried out using a paper disc diffusion method. After removing mycelia from the S. carzinostaticus ATCC15944 wild-type or recombinant strain culture broth by centrifugation, the resultant supernatant was then fractionated by addition of (NH4)2SO4, and NCS chromoprotein was precipitated between 40%–85% saturation. The precipitated NCS chromoprotein was collected by centrifugation, dissolved in 5 volumes of 15 mM NaOAc (pH 4.5) buffer, and dialyzed against H2O at 4°C. The dialyzed sample was freeze-dried and NCS chromophore was extracted with MeOH. The isolated yield for the NCS chromophore from the wild-type strain by this method varies between 1–5 mg/l.

Bioassay of the chromophore obtained from the recombinant strain was performed on an agar plate containing M. luteus grown overnight in LB medium. Alternatively, agar plugs of S. carzinostaticus ATCC15944 wild-type or recombinant strain grown on solid media were placed directly on M. luteus seeded LB plates. The plate was incubated at 37°C for 24 hr and NCS chromoprotein concentration was estimated by the size of the growth inhibition zone.

Supplemental Data

Supplemental Data for this article is available online at http://www.chembiol.com/cgi/content/full/12/3/293/DC1/.

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References


Accession Numbers

The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number AY117439.